STUDIES ON THE CONNECTIVE TISSUE MATRIX COMPONENTS OF THE ARTERIAL WALL OF HUMANS AND SALMONIDS (SALMO GAIRDNERI AND SALMO SALAR): AGE-AND SPECIES-RELATED PHYSICAL AND CHEMICAL PROPERTIES

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A thesis presented by

MICHELE SPINA

to

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ACADEMIC RECORD

I first matriculated at the University of Padova, Italy, in October, 1959 and graduated with the degree of Doctor of Medicine in December, 1967.

I matriculated as a research student in the Department of Biochemistry, University of St.Andrews in Novenber 1976.

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that it is my own composition, and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Prof. Augusto Serafini-Fracassini, and in the Istituto di Istologia ed Embriologia Generale, University of Padova, under the direction of Prof. Lorenzo Gotte.

CERTIFICATE

It is certified that Michele Spina has spent eleven terms engaged in research work under the direction of Prof. Augusto Serafini-Fracassini, Department of Biochemistry and Microbiology, University of St.Andrews, and Prof. Lorenzo Gotte, Istituto di Istologia ed Embriologia Generale, University of Padova, and that he has fulfilled the conditions of Ordinance General No. 12, and Resolution of the University Court, 1967, No. 1, and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Prof. Lorenzo Gotte
Head of Institute

Prof. Augusto Serafini-Fracassini

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This work is dedicated to my father, Ribelle Spina.

S U M M A R Y

Section A of this thesis describes the non-degradative isolation procedure of elastin from the media of human thoracic aorta and the determination of its ponderal distribution as well as that of the other connective tissue matrix components in the aortic media of individuals of different ages. A cylindrical segment, free of complex atherosclerotic lesions, was resected at autopsy from each of fifty-nine descending human thoracic aortae by cutting just below the level of the first pair of intercostal arteries and 35 mm distal to this incision. In the isolated tunica media there was an age-related rise in the absolute concentration of the following components:
(a) proteins and glycoproteins extractable in chaotropic agents, (b) collagen and (c) an insoluble polar protein(s) which was resistant to collagenase digestion but was solubilized by trypsin. In contrast, the absolute elastin concentration was constant at around 70 mg/cm in samples of all ages. Although after treatment with collagenase and trypsin, both the mechanical properties and amino acid composition of elastin did not vary with age, in samples tested prior to trypsin digestion the insoluble polar-protein contaminant affected the mechanical behaviour of the elastin network in a way akin to plasticisers in rubber. It is therefore suggested that the morphological changes and stiffening observed in the ageing aortic wall are not due to degradation of its elastin network bu to variations in the supramolecular organisation of connective tissue components.

Section B of this thesis describes the isolation of elastin and the determination of its distribution as well as that of collagen in the bulbus arteriosus and ventral aorta of trout (Salmo gairdneri) and salmon (Salmo salar). The isolation of elastin fibrils from these specimens did not require the use of proteolytic enzymzes as collagen and all the other components of the vessel wall were readily extracted in denaturing and reducing conditions. The arterial walls of trout and salmon are characterized by a high elastin content, with an elastin:collagen ratio exceeding 2.5:1. The elastin which display an amino acid composition highly at variance with that of the mammalian protein, is present as 250-Å-diameter fibrils which have a strong affinity for electron-dense cations and which form in the intercellular matrix an isotropic reticulum. As in mammalian elastin, the fibrils can be resolved in negatively contrasted preparations into constituent primary filaments of about 25 Å in diameter, aligned with an equatorial periodicity of about 55 Å. The wide-angle X-ray diffraction pattern of the salmonid preparations showed broad reflections corresponding to spacings of 9.8, 4.5 and 2.2 Å, similar to bovine elastin. The mechanical behaviour of the salmon preparation was characterized by a linear response to stress, with minimal hysteresis, a Young's modulus of 5.5×10^5 N m^{-2}, and a breaking strain of 1.5.

CONTENTS

RESULTS

STUDIES ON THE CONNECTIVE TISSUE MATRIX COMPONENTS OF THE ARTERIAL WALL OF HUMANS AND SALMONIDS (*Salmo gairdneri* and *Salmo salar*): AGE- AND SPECIES-RELATED PHYSICAL AND CHEMICAL PROPERTIES

ABBREVIATIONS

Guan-HCl :	Guanidine hydrochloride
TRIS :	Tris(hydroxymethyl)aminomethane
EDTA :	Ethylenediaminetetra-acetic acid
DTE :	Dithioerythritol; 2,3-dihydroxy-1,4-dithiolbutane
DPCC :	Diphenyl carbamyl chloride
SDS :	Sodium dodecyl sulfate
TR :	Traces
ND :	Not determined
SD :	Standard deviation
SE :	Standard error
NS :	Not significant

I N T R O D U C T I O N

The mechanical properties of arteries are an important determinant of hemodynamics. The arterial side of the circulatory system is not only a simple conducting system, but also an elastic buffering chamber (1). In humans the aorta, pulmonary artery and large distributing arteries are distended rapidly during ventricular ejection, transiently accomodating 50% or more of the stroke volume and storing the energy which enables them to force the blood along by elastic recoil during diastole (2, 3). In mammals positive elastic flow in the aorta prevails for less than one-third of the cardiac cycle (4). In fishes, positive flow in the ventral aorta may persist through more than three-fourths of the cardiac cycle (5). These blood vessels owe their peculiar properties to the presence of the protein elastin in their walls.

Elastic tissue is responsible for the long-range, reversible extensibility of arteries, lungs, ligamenta flava of the vertebrae and other physiologically important connective tissues. It is found in two characteristic morphological forms depending upon its anatomical location. In bovine ligamentum nuchae longitudinally oriented elastic fibres of 4-7 μm in diameter form the predominant element (6) while in blood vessels elastic tissue is present mainly as thin fenestrated sheets (lamellae) concentrically arranged with respect to the main axis of the vessel (7, 8). The lamellae are composed of fibrillar aggregates of varying size, which run predominantly in a circumferential direction (7). Elastic fibres are embedded in an amorphous matrix in close association with collagen fibrils which occupy zones between elastic fibres (8). Examined by electron microscopy in stained sections of mammalian tissues, elastic fibres have been shown to consist of two morphologically distinct components, namely an amorphous elastin core and a surrounding layer of microfibrils which are approximately 11 nm in diameter (9). By scanning electron microscopy the amorphous elastin core can be further resolved into 120-nm-diameter fibrils (6, 10) and negatively contrasted preparations of purified elastin reveal the presence of beaded filaments of about 2.5-4.0 nm diameter, approximately parallel to the long fibre axis (11, 12, 13, 14, 15). High resolution electron micrographs suggest that each beaded filament consisted of paired subfilaments of about 1.5 nm in diameter (15).

Elastin is mechanically similar to most polymeric rubbers (17). In connective tissues it provides a low modulus, elastic element that can be deformed to large strains and then will restore the tissue to its initial unstrained dimensions. Chemically it is characterized by the presence of lysine-derived polyfunctional amino acids, namely, desmosine, isodesmosine, merodesmosine, lysinonorleucine and the aldol condensation product which crosslink polypeptide chains and impart to the protein its ability to sustain deformation without chain slippage (18, 19, 20, 21).

Mammalian elastin has an unusually high content of non-polar amino acid residues, with a prevalence of glycine, alanine, valine and leucine, a low content of polar amino acids and a small amount of hydroxyproline (22, 23). The non-polar nature of elastin is further emphasized by the fact that 80% of the aspartic and glutamic acid residues are amidated (24). Recent studies on the phylogenesis of aortic elastin have indicated that proteins containing the polyfunctional amino acids characteristic of mammalian elastin occur in all vertebrate groups with the exception of the cyclostomes and that these proteins differ greatly in overall amino acid composition as well as the total concentration and relative proportion of lysine-derived crosslinks. These findings have led to the suggestion that elastin has the potential to undergo rapid evolutionary change (25, 26). Of these elastins, that present in the vascular system of Salmonids shows the lowest content of crosslinks and an amino acid profile greatly at variance with that of mammalian preparations.

The high content of covalent crosslinks in mammalian elastin renders it highly insoluble and therefore isolation of this protein from connective tissue matrices must rely upon removal of all other tissue components. Isolation procedures have taken advantage of elastin's relative stability toward weak acid (27), hot dilute alkali (28) and autoclaving in water (29), and to date the most widely accepted criterion for the assessment of an elastin preparation's purity has been the comparison of its amino acid composition with that of the insoluble residue obtained after treatment with 0.1 N NaOH at 98°C for 45 minutes (22). However, although this procedure is successful in the purification of elastin from bovine ligamentum nuchae and aorta it does not produce acceptable preparations from uterine or cartilaginous tissue (30, 31, 22), while elastin obtained in this way from old human lung and aortic tissue specimens is contaminated with a more polar protein or group of proteins (32, 33, 34, 35) which can only be removed from the elastin residue if the samples are autoclaved and decalcified with EDTA prior to the hot alkali treatment (36). It is worth noting that the demonstration of extensive peptide bond cleavage in hot alkali purified elastin from bovine and human aorta (37, 38) as well as the lability of serine and threonine in such conditions (39) greatly affect the reliability of this purification procedure when conformational and compositional parameters are to be investigated.

In order to overcome these difficulties, many alternative purification procedures have been devised (23, 40). However, the only method which avoids extremes of temperature and pH is that based on successive modifications of the procedure of Miller and Fullmer (41) in which contaminating structural components are selectively extracted, or hydrolyzed by specific enzymes (9, 42, 43). The consistent amino acid composition and low levels of N-terminal residues detected in such preparations has led to the conclusion that enzymic purification is effective in the isolation of elastin from bovine ligamentum nuchae and aorta, while their close compositional similarity to soluble tropoelastin suggests that these preparations have a higher degree of purity than alkali insoluble elastin (37, 42).

Although elastin in mature elastic fibres exists as an insoluble crosslinked protein, the biosynthesis of elastic tissue starts with a soluble precursor tropoelastin which is synthesized inside the cell, transported to the extracellular space and then crosslinked into elastin network. In copper-deficient or lathyric animals, the first step in the biosynthesis of crosslinks, namely the oxidation of lysyl residues by the enzyme lysyl oxidase (44), is inhibited. This allow tropoelastin or salt soluble elastin to be isolated from aortae of copper-deficient swine (46). It comprises some 850 amino acids and has a molecular weight of 74000 daltons (47, 48). A precursor-product relationship between tropoelastin and elastic fibres is suggested by the similarity in their amino acid composition, the absence of crosslinks and higher number of lysine residues in tropoelastin, the presence of tropoelastin in organ culture systems and the fact that tropoelastin has coacervation properties similar to that of the alfa-elastin described by Partridge (29, 49, 50, 51, 52, 53). In addition neither protein contains methionine, cystine, tryptophan or histidine, while tropoelastin has also been isolated from elastic tissue such as chick aorta (54) and from bovine ligamentum nuchae (55). It is interesting to note that aortic smooth muscle cells have been shown to synthesize tropoelastin (56). Table 1 shows the amino acid composition of insoluble elastins isolated from animals fed at normal diet and the amino acid composition of the corresponding tropoelastins isolated from lathyric and copper-deficient animals.

The main post-translational modifications of tropoelastin include hydroxylation of proline and oxidative deamination of lysine residues. The conversion of lysine in tropoelastin to allysine by the enzyme lysyl oxidase is required for the synthesis of intermolecular crosslinks, and oxidative deamination of the ε-amino group of selected lysine residues yields residues of α-amino adipic δ-semialdehyde (allysine) which can then participate in Schiff-base reactions with ε-amino groups of other lysine residues or in aldol condensation with other aldehydic residues

TABLE 1

Amino acid composition of insoluble elastins purified from normal animals and tropoelastins isolated from copper-deficient (§) and lathyric (ι) animals.

Results are expressed as amino acid residues/1000 residues.

TABLE 1

Source	Bovine				Porcine				Chicken		
	Insoluble elastin			Tropoelastin	Insoluble Elastin	Tropoelastin			Insoluble elastin		Tropoelastin
	Ligamentum nuchae	Aorta	Ear	Ligamentum nuchae (§)	Aorta	Aorta (§)	Aorta (ı)	Ear (ı)	Aorta	Lung	Aorta (§)
Reference	(42)	(37)	(43)	(55)	(76)	(76)	(116)		(23)		(54)
Amino acid											
Hydroxyproline	8.5	10.6	11.7	8	10	4	10	20	26	18	9
Aspartic acid	5.7	6.5	21.4	6	4	3	10	6	7	11	8
Threonine	9.2	9.6	12.9	8	12	9	16	15	9	11	9
Serine	8.6	9.2	14.8	9	9	8	15	13	6	11	15
Glutamic acid	15.1	16.0	35.7	15	18	11	18	21	13	18	18
Proline	114.0	112.7	113.7	92	122	116	107	101	131	120	143
Glycine	336.2	332.4	307.3	316	326	326	320	327	338	336	338
Alanine	224.0	223.9	204.3	220	230	274	230	222	179	185	175
Valine	129.9	131.3	105.0	147	131	120	122	123	173	164	143
Half-cystine	0.0	0.0	0.0	0	ND	ND	ND	ND	ND	ND	0
Methionine	0.0	0.0	0.0	0	ND	ND	0	0	ND	ND	0
Isoleucine	23.6	23.5	19.9	20	19	11	15	18	20	19	16
Leucine	58.6	58.2	62.2	55	54	38	49	45	56	58	48
Tyrosine	5.8	7.5	16.0	6	17	10	16	13	13	13	11
Phenylalanine	28.9	29.7	29.3	32	32	21	29	29	19	21	17
Hydroxylysine	0.0	0.0	0.0	0	ND	ND	ND	ND	ND	ND	ND
Lysine	3.2	4.6	7.3	49	5	39	38	30	2	6	44
Histidine	0.4	0.5	0.7	Trace	0.3	1	2	2	0.7	1	0
Arginine	5.7	5.9	14.3	5	6	4	7	7	5	6	5
ε-Hydroxynorleucine	0.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aldol condensation product*	0.6	ND	5.7	ND	ND	ND	ND	ND	ND	ND	ND
Dehydrolysinonorleucine*	0.0	ND	0.0	ND	ND	ND	ND	ND	ND	ND	ND
Lysinonorleucine*	2.1	2.1	2.1	ND	0.4	0	ND	ND	ND	ND	ND
Dehydromerodesmosine*	0.1	ND	0.7	ND	ND	ND	ND	ND	ND	ND	ND
Merodesmosine*	0.5	0.6	Trace	ND	0.6	0	ND	ND	ND	ND	ND
Isodesmosine*	5.4	5.5	4.9	0	3.6	0	0	0	4.8	5.6	0
Desmosine*	10.1	9.6	10.4	0	2.4	0	0	0	5.6	6.4	0
Amide	16.6	ND	43.5	ND	ND	ND	ND	ND	ND	ND	ND

*Expressed as lysine equivalents (§)Copper deficient (ı)Lathyric ND Not determined

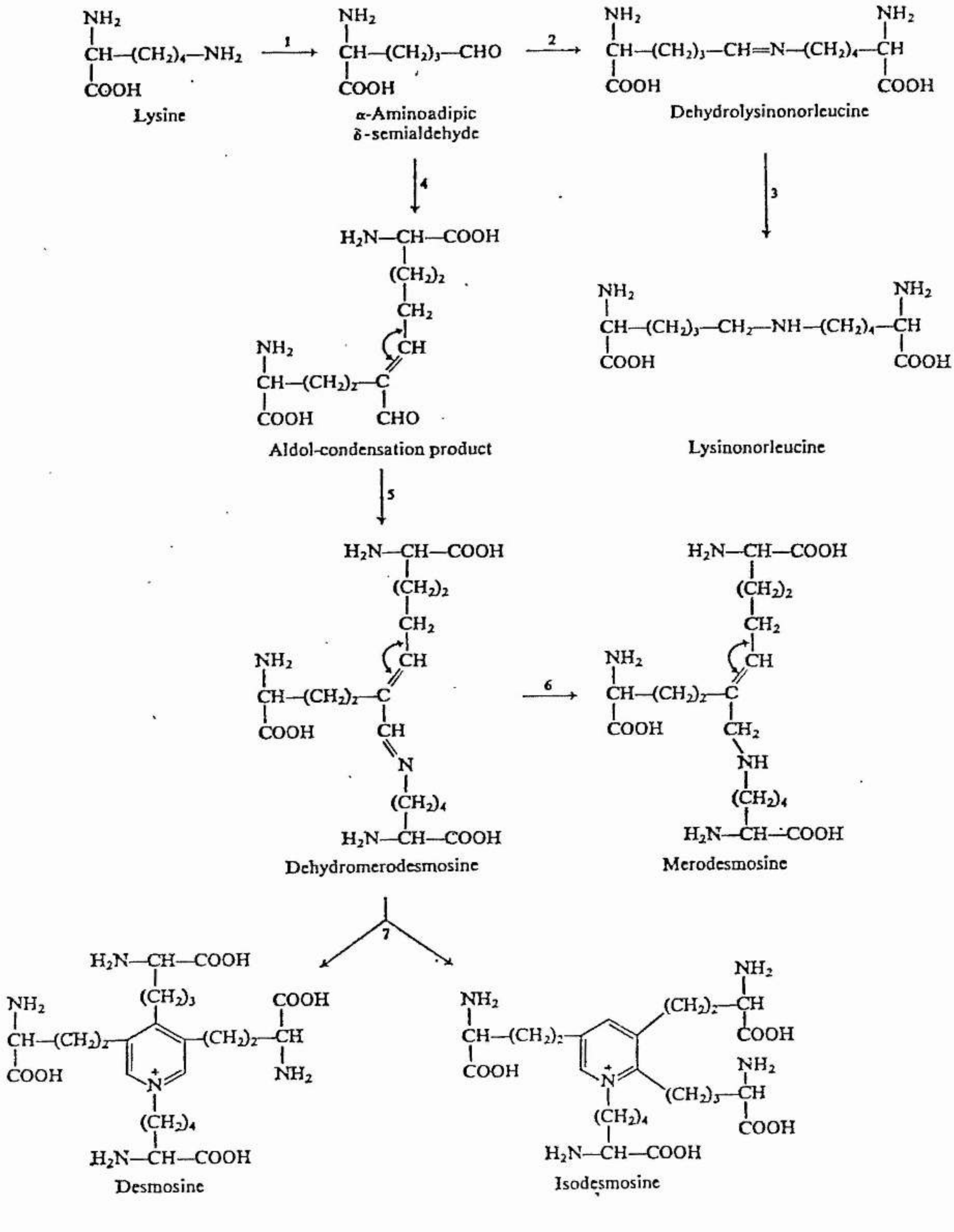
(44). The first two crosslinking compounds to be isolated from elastin, desmosine and isodesmosine, have been shown to be 1,3,4,5, and 1,2,3,5,-tetrasubstituted pyridinium salts, respectively (18, 59), which both exhibit a characteristic ultraviolet absorption spectrum with a maximum at 275 nm. The side-chain at position 1 on the pyridinium ring contains six carbon atoms in both isomers (18, 60) while the length of the other three side-chains depends upon the position of substitution on the ring. Later less complex lysine-derived crosslinking compounds have been discovered and the structure of these compounds as well as the possible routes of their biosynthesis from lysine residues are presented in Table 2, as proposed by John and Thomas (58).

The crosslinking compound lysinonorleucine is designated N^{ε} (5-amino-5-carboxypentyl) lysine and was first isolated from acid hydrolysates of elastin. It was shown to be produced by reduction "in vivo" of a Schiff-base precursor (dehydrolysinonorleucine) formed by the interaction of a residue of the semialdehyde with a lysine residue (Table 2, reaction 2 and 3). Similarly, the product derived from the aldol condensation of two semialdehyde residues, the so-called aldol-condensation product (reaction 4, Table 2) was isolated as the corresponding alcohol derivative from the alkaline hydrolysates of elastin that had been first treated with the reducing agent $NaBH_4$ (21). A more complex compound, merodesmosine, derived from three lysine residues, is probably formed from its reducible precursor dehydromerodesmosine, an acid labile compound resulting from the interaction of the aldol-condensation product and the

TABLE 2

Structures of lysine-derived crosslinks in elastin showing their possible synthesis from lysine molecules. In elastin the α-amino and α-carboxyl groups form peptide bonds. From reference (58).

Table 2



Lysine
α-Aminoadipic δ-semialdehyde
Dehydrolysinonorleucine
Aldol-condensation product
Lysinonorleucine
Dehydromerodesmosine
Merodesmosine
Desmosine
Isodesmosine

ε-amino group of a third lysine residue (19),(Table 2).

Recently a high molecular weight form of soluble elastin of 130000 molecular weight which undergoes degradation yielding tropoelastin and a more acidic component, has been isolated from aortae of lathyric chicks (56, 57), and it has been postulated that it might represent the true primary precursor of insoluble elastin. It was designated as proelastin and suggested that it might be incorporated into the elastic fibre without being converted to tropoelastin.

The sequencing of peptides produced by tryptic cleavage of tropoelastin has demonstrated the existence of two distinct regions. A group of small peptides comprising four residues or less accounts for half of those produced by tryptic cleavage (61) and are presumably derived from the regions which are involved in crosslinking (62, 63). On a molar basis there seem to be six peptides with the structure Ala-Ala-Ala-Lys and six with the structure Ala-Ala-Lys. There are two peptides with each of the following structures: Ser-Ala-Lys, Ala-Pro-Gly-Lys and Tyr-Gly-Ala-Arg, and at least one dipeptide with the structure Ala-Lys.

The second set of tryptic peptides are much larger, having an average size of 50 residues (62, 64). These represent the portions of the polypeptide chain that lie between crosslink areas and presumably are responsible for the elasticity of the protein. The N-terminal peptide contains four copies of the sequence Gly-Gly-Val-Pro which also appears sporadically elsewhere. In the other tryptic peptides the pentamer Val-Pro-Gly-Val-Gly and the hexamer Ala-Pro-Gly-Val-Gly-Val appear as the most striking structures as they occur six and five times respectively in continuous sequences at one particular point and also as scattered repeats elsewhere in the molecule. It is interesting to note that a peptide (7-8000 daltons) derived from insoluble elastin has been shown to be essentially a multimer of the pentamer mentioned above (65).

The general organization of the cardiovascular system in humans is well known.

The systemic heart of fishes consists of four chambers in series, the sinus venosus, atrium, ventricle, and conus arteriosus (elasmobranchs) or bulbus arteriosus (teleosts) (66). All chambers except the bulbus arteriosus are normally contractile and are separated by valves which maintain a unidirectional flow of blood through the heart into the ventral aorta.

Many investigations of the teleosts heart have demonstrated an important regulatory role of both the bulbus arteriosus and ventral aorta in effecting maintenance of blood flow in late systole and far into ventricular diastole (67). In the trout (Salmo gairdneri) and in lingcod (Ophiodon elongatus) 25% and 29% of the blood flow, respectively, is due to the elastic rebound of the bulbus arteriosus (68, 69). In humans systolic and diastolic blood pressure is more than twice as high as that in salmonids and in most other teleosts (5). However in spite of the lower blood pressure in teleosts, the bulbus arteriosus appears to be distended many times more by the pulse wave than the human aorta. The values recorded in human ascending aorta for radial dilatation with pulse pressure wave account for a circumferential strain of 0.11 (70), while in the mid-bulbus arteriosus in the eel (Anguilla anguilla) a circumferential strain of 0.67 has been measured (71). When a body of length L_o is extended to a length L_1, then the relative increase in length $(L_1 - L_o)/L_o$ is the longitudinal strain.

The mechanical characteristics of blood vessels are determined by both passive and active tissue components. The most significant passive components are the fibrous connective tissues: elastin and collagen. Proteoglycans and glycoproteins are a quantitatively minor component in the arterial walls (72). The active contribution to the arterial mechanics is given by the smooth muscle and it varies in different types of vessel (73). In the smaller arteries (muscular type) and arterioles, contraction of vascular muscle causes large changes in the vascular lumen with corresponding changes in the periferal resistance and blood pressure. In larger arteries (elastic type) the stimulation of smooth muscle does not produce any obvious changes in the size of the lumen, but it can raise the tension on the elastic tissue and thus affect the blood pressure. While relaxed muscle develops virtually no tension until it is stretched to great lengths, at maximal contraction vascular muscle can exert a force of 1-3 x 10^6 dyn/cm^2 or possibly higher (74).

Three layers can be distinguished in the wall of every artery (75):
1) The inner coat, tunica intima, which comprises a single continuous layer of flat endothelial cells and a thin sub-endothelial layer consisting of ground substance, a few collagen fibres and fibroblasts.
2) The intermediate coat, tunica media, contains elastin, collagen, smooth muscle cells and groud substance. Particularly in the human thoracic aorta

light and electron microscopic studies indicate that medial structural components are arranged in an orderly fashion: concentric fibrillar elastin lamellae are connected by an intricate network of elastin fibrils with interspersed collagen fibres and smooth muscle cells (77, 78, 79, 80). The overall static mechanical properties of the vessel have been considered to be dependent mainly on the concentration and interrelation of medial components.

3) The external coat, tunica adventitia, is composed of loose connective tissue with longitudinally arranged collagenous and elastic fibres, and few fibroblasts.

In the bulbus arteriosus of teleosts, particularly in the carp (81), the tunica adventitia and the tunica intima are very thin. The tunica media occupies more than 90% of the unstretched bulbar wall and is much thicker in proportion to the vessel wall than is the tunica media of mammalian elastic arteries. Under histological examination almost all the collagen fibres appear to be confined to the adventitial layer while the media comprises smooth muscle and elastic tissue in intimate contact. In King salmon (Onchorhynchus tshawytscha) bulbus arteriosus, 30% of the total protein content is accounted for by elastin (26), while in human aortic arch elastin accounts for 38% of the dry defatted weight (72).

While the distensibility of the arterial wall is determined largely by the compliance of the elastin, much of the stiffness of arteries, especially at very large distensions, is attributable to collagen which is rather inextensible and bears the stress when pressure becomes high, protecting the vessel from rupture or blow out (73, 82). Pure collagen fibres can be extended to strain of only 0.03-0.04 before breaking (83, 84), while bovine elastic fibres undergo rupture at a strain of 0.80 (85). It has recently been reported that the Young's modulus of collagen would fall in the range 0.90-2.15 x 10^{11} dyn/cm^2 (86), while that of fully hydrated elastic fibres from ox ligamentum nuchae has the value 10^7 dyn/cm^2 (87). At distending pressure of 100 mm Hg the elastic modulus of the thoracic aorta is at least twice great as that of purified elastin (88), isolated from bovine ligamentum nuchae. Therefore it has been suggested that both the elastin and collagen of the aortic wall are stressed over a wide range of distending pressure, particularly within the physiological range (89). Nevertheless the quantitative contribution of each of these components to the arterial wall mechanics remains to be determined.

There are few reported measurements of the individual contribution of vascular collagen and elastin to arterial deformability in humans (27, 78, 89). However, the absence of analytical data regarding the purity of the samples, the use of crude proteolytic enzyme preparations and the exposure to extremes of temperature and pH during the purification procedure seriously affect the reliability of the mechanical parameters derived from these preparations. No correlation has been found between collagen content of human aortic specimens and the stiffness that appeared in distended vessel specimens (90).

With age the human aorta dilates and becomes functionally stiffer at physiological pressures (3, 91, 92). The impairement in function of the artery has been related to a substantial increase of the collagen content (diffuse fibrosis) (93) and to a decrease of elastin in the vessel wall (94). Thinning, straightening, fragmentation and calcification of elastic lamellae in the aortic media increase with advancing age (95, 96, 97, 98, 99). More severe alterations of the elastic tissue have been found focally at the intima-media region where massive calcification occurs (100, 101, 102).

Studies on the fibrous protein compositional changes occurring concomitantly with these morphological changes during aging have given conflicting results. Some authors have reported a decrease in elastin and an increase of collagen content in media-intima preparations (103, 104), others have shown that the concentration of elastin and collagen in the aortic media remains constant independent of age (96, 105, 106). These conflicting reports probably reflect variations in methodology, sampling and the use of the relative content rather than the absolute content of each component to express results (107). In fact in mammals the total mass of aorta increase with age (108, 109, 110, 111), and it has been noted that age-related processes such as the impaired function (112, 113) and the calcification of the elastic fibres in the media (99, 105, 106, 114), should possibly be considered as independent from those occurring in atheromatous areas in the intima. It is interesting to note that a pronounced cranio-caudal fall in the relative concentration of elastin has been found in the aorta of mammals, while the relative concentration of collagen varied inversely (72, 115).

The aims of this work have been:

a) the isolation of elastin by mild procedure from human thoracic aorta, bulbus arteriosus and ventral aorta of two closely related teleosts (salmon and trout) in order to study some of the chemical and physical properties of these preparations;

b) a reinvestigation of the age-related ponderal distribution of collagen, elastin and other components in the human aortic media, following more precise anatomical and comparative criteria in sampling and data evaluation;

c) the determination of the concentration of these connective tissue matrix components in the arterial vessels of trout and salmon;

d) a tentative evaluation of the relationship existing between the amino acid composition, the number of crosslinks in elastins purified from different sources and some of their physical properties.

While human aorta becomes functionally stiffer with age, there is little information about the possible age-related mechanical changes in the elastin network isolated by non-degradative procedure from the media of this vessel. According to Hass who performed stress-strain measurements on elastic fibres isolated by hot formic acid from human aortic rings of individuals of different age, "if cross-sectional areas are adjusted to a standard value, there is close correspondence of the slopes of all curves" (27).

On the other hand it is not known whether the larger compliance observed in teleosts arterial vessels as compared to that found in human aorta has to be related to the presence of a lowly crosslinked and more compliant elastin or to a different distribution of the other tissue matrix components. In fact desmosine and isodesmosine content is markedly low in teleost elastin (81) as compared to the mammalian elastin, but the concentration of the other crosslinks deriving from the reaction between two and three lysine residues has not yet been determined in these preparations.

Section A of this thesis describes the non-degradative isolation procedure of elastin from the media of human thoracic aorta and the determination of its ponderal distribution as well as that of the other connective tissue matrix components in the aortic media of individuals of different ages. The content of each single component in the aortic media has been expressed by mean of its relative per cent concentration (w/w) with reference to the dry defatted decalcified weight and by its absolute amount expressed in mg found in one unit length (cm) of the hydrated isolated media. According to a modified version of the Ross and Bornstein (9) procedure, the tissues were defatted with organic solvents, decalcified in 0.2 M EDTA / 0.4 M TRIS pH 7.4, extracted with 5 M Guan-HCl, followed by digestion with bacterial collagenase (EC 3.4.24.3) purified by affinity chromatography (42). As the insoluble residue obtained following this procedure was still contaminated by polar protein components, the preparation was further digested with trypsin (EC 3.4.21.4) which does not degrade fibrous elastin as previously reported (23, 58). Both stress-strain experiments and amino acid analyses of the insoluble elastin residue were carried out before and after the final step of the purification procedure.

Section B of this thesis describes the isolation of elastin and the determination of its distribution as well as that of collagen in the bulbus arteriosus and ventral aorta of trout (Salmo gairdneri) and salmon (Salmo salar). The isolation of elastin fibrils from these specimens did not require the use of proteolytic enzymes as collagen and all the other components of the vessel wall were readily extracted in denaturing and reducing conditions, according to the above mentioned procedure.

The insoluble elastin fibrils were examined by transmission electron microscopy, X-ray diffraction analysis and dynamometry. The amino acid composition and the whole pattern of crosslinks and crosslink-precursors was determined on acid and alkaline hydrolysates while fluorescence optical properties were studied on elastase (EC 3.4.21.11) digests.

SECTION A

MATERIALS AND METHODS

M A T E R I A L S

Fifty-nine samples of whole descending human aorta were obtained fresh at autopsy from the Istituto di Anatomia Patologica, Padova, Italy. Specimens with complex atherosclerotic lesions were not collected. The ages of the individuals providing the samples ranged from 19 to 78 years. Collagenase (EC 3.4.24.3, from Clostridium histolyticum, Type I), DPCC-trypsin (EC 3.4.21.4, from bovine pancreas, diphenyl carbamyl chloride treated, Type XI), dithioerythritol (DTE; 2,3-dihydroxy-1,4-dithiolbutane), amino acid standard solutions, d-glucosamine hydrochloride, d-galactosamine hydrochloride, d-glucuronic acid lactone, were purchased from Sigma, Cemical Co., St. Louis, Mo, USA. Guanidine hydrochloride (analytical grade) was obtained from Carlo Erba, Milano, Italy. All other reagents were analytical grade and were obtained from Merck, Darmstadt, and used without further purification.

METHODS

Sampling of human aortic segments

The aortae were washed with cold saline and after a large part of the adventitia had been removed, the vessels were resected just below the level of the first intercostal artery pair following a plane orthogonal to the main vessel axis. The proximal segment was set aside, while the distal segment was resected transversely approximately 35 mm distally to the former resection plane. The segment of the vessel between these two resection levels is anatomically defined as upper thoracic aorta and was considered for the present study. The cylindrical segment was chosen for two reasons, firstly it is straight and regular in shape and so the determination of the lateral surface area can be carried out following simple geometrical calculations, secondly the adventitia can be completely removed by dissection along the plane of cleavage at the junction of the media and the adventitia (117).

Ten upper thoracic aortic segments were isolated for each of six age groups corresponding to a decade, with the exception of the 30-39 years group which consisted of eight samples. One aortic specimen from a 19 year old subject was processed separately.

The mean age calculated for each of the six groups corresponded to 23.6, 33.9, 45.1, 54.7, 63.2 and 73.2 years respectively. Within each group one sample, selected at random, was labelled and its age recorded. The six selected specimens were taken from 29, 39, 44, 56, 65 and 76 year old sujects. The other samples in each group were numbered, but their age was not recorded.

Preparation of the aortic segments

Using the procedure of Wilens (112) the intima and the remaining adventitial connective tissue were dissected away with scissors and forceps and discarded. The resulting medial segments were hung on a glass framework, immersed in organic solvents and defatted by stirring the surrounding fluid at 4 °C for successive 24 h periods with acetone, chloroform/methanol 2/1 (v/v), chloroform/methanol 3/1 (v/v), absolute ethanol and di-ethyl ether. Hanging of the samples on a glass framework was adopted in order to avoid mechanical damage to the samples following collision with the magnetic flea. Ether was removed in an air stream and the samples were stored under reduced pressure for 48 h over P_2O_5 and weighed. After a suitable number of samples had been collected, the specimens were rehydrated overnight at 4 °C in distilled water, blotted with filter paper and weighed. The external circumference of both proximal and distal end (with reference to the distance from the heart) as well as the length of each rehydrated segment was measured (Figure 1).

Sequential extraction of the defatted aortic segments

The defatted rehydrated samples of each age group were extracted (a) for three 24 h periods with 600 ml of 0.2 M EDTA / 0.4 M TRIS (pH 7.4) at 4 °C with continuous stirring of the surrounding fluid, to remove the mineral phase and associated glycoproteins (118). This treatment was followed by (b) six 24 h extractions in 600 ml of 5.0 M Guan-HCl / 0.4% (w/w) EDTA / 0.1 M TRIS (pH 7.4), conditions which ensured the solubilization of muscle proteins, proteoglycans and glycoproteins of the ground substance (119). In order to remove the microfibrillar component associated with elastin in mammalian tissues (9), each group of segments was further extracted (c) for two 24 h periods in 300 ml of 50 mM DTE / 5.0 M Guan-HCl / 0.4% (w/w) EDTA / 0.1 M TRIS (pH 7.6) at 37 °C under nitrogen. At the end of each group of extractions (a, b, c) the residual segments were washed for 12 h periods with several changes of 2.0 M NaCl and distilled water (42), dried with absolute ethanol and ether, stored under reduced pressure over P_2O_5 and weighed. The dry mass distribution within the wall of both defatted and sequentially extracted residual medial segments was expressed as mg/cm and mg/cm^2 according to the ratio of the dry weight to the length and to the lateral surface area, respectively, shown by each segment after its initial defatting and successive rehydration. For each age group the data have been expressed as the mean $\pm$ SE of the values determined on each segment (Figure 1 and 2).

The total amount of material solubilized by each treatment was estimated from the difference between the dry weight of each aortic segment before and after the corresponding extraction. The supernatants of each group of extractions (a, b, c) were pooled separately, filtered through a millipore membrane, dialyzed for a week against H_2O at 4 °C with several changes of the external fluid, lyophilized, dried under reduced pressure over P_2O_5 and weighed. Prior to dialysis the supernatants of the (c) group of extractions were S-carboxymethylated by addition of sodium iodoacetate in a fourfold molar excess over DTE, the pH was adjusted and maintained at

8.6, and the reaction mixture stirred for 45 minutes under nitrogen. After addition of a five-fold molar excess of mercaptoethanol over iodoacetate, the solution was dialyzed against water for a week. The amount of undiffusible material was evaluated by determining protein concentration from recovery of amino acids after chromatographic analysis and from total carbohydrate content, following estimation of hexoses, hexosamines and uronic acids (see below).

Sampling of aortic rings and fragments

After sequential extraction, each of the six segments of known age, which had been labelled prior to defatting, was divided transversely into a series of cylindrical rings which varied from 4 to 5 mm in height. The rings corresponding to the proximal and the distal ends of each segment were labelled Proximal and Distal, respectively, while those corresponding to the middle portion were labelled Interm. The Proximal, Distal and Interm rings were used for the enzymic and hot alkali preparation of insoluble elastin and for physical studies. Circumferential fragments of equal weight were excized from the proximal and the distal ends of all the other segments (after sequential extraction), pooled according to age group and set aside for amino acid analysis. The dry weight of the aortic rings and pooled circumferential fragments was determined.

Isolation of the insoluble elastin with enzymes

Each Proximal, Distal and Interm ring was separately suspended in 20 ml of 10 mM $CaCl_2$ / TRIS 0.1 M (pH 7.5) and equilibrated for 18 h at 4 °C. The samples were then resuspended in fresh buffer, the temperature raised to 37 °C and bacterial collagenase (EC 3.4.24.3), purified by affinity chromatography (42) and dissolved in 5 ml of the same buffer was added at an enzyme/substrate ratio 1/1000. At the end of each of three 24 h digestion periods the rings were washed with several changes of buffer for 6 h in each of the following solutions; Guan-HCl 5.0 M / TRIS 0.1 M (pH 7.5), NaCl 2.0 M and distilled water. The rings were then dried as before and weighed. This material will be referred to as collagenase-purified fibres. Proximal and Distal rings were set aside for physical studies, while Interm rings were further equilibrated for 18 h at 4 °C in 20 ml 1% (w/v) NH_4HCO_3 (pH 8.0) and digested with DPCC-trypsin (EC 3.4.21.4) at an enzyme/substrate ratio of 1/10 for 24 h at 37 °C. The residual rings were washed as outlined above, dried and weighed. This material will be referred to as trypsin-purified elastin fibres.

Treatment of collagenase-purified elastin fibres with hot alkali

After performing the mechanical test each collagenase-treated Proximal and Distal ring was transversely divided in two halves and dried and weighed as before. One half was subjected to acid hydrolysis for amino acid analysis and the other half was suspended in 0.1 N NaOH and refluxed for 45 minutes (28). Then the residual fragment was transferred in distilled water, neutralized with dilute HCl, washed with Gúan-HCl, NaCl, distilled water, dried and weighed. This material will be referred to as alkali-treated elastin.

Evaluation of the relative and absolute concentration of connective tissue matrix components within the isolated media of defatted aortic segments

The relative concentration (w/w) of aortic medial tissue components has been evaluated with reference to the dry weight of the defatted decalcified samples of each group. The absolute concentration has been expressed as mg/cm (dry weight/wet length) and as mg/cm^2 (dry weight/wet lateral surface area) for the defatted rehydrated segments of each age group before their extraction with EDTA.

The total amount of material solubilized during the treatment with Guan-HCl/TRIS and Guan-HCl/TRIS/DTE has been reported in Table 11 as the mean of the values determined gravimetrically on every segment of each age group.

Elastin concentration has been determined both gravimetrically on the insoluble residue remaining after treating the Interm rings with collagenase followed by trypsin and chemically according to the amino acid composition of the sequentially extracted pooled fragments (Table 7). In the latter instance elastin concentration has been calculated with reference to the average content of desmosine and isodesmosine found in trypsin-purified elastin fibres (Table 9) which were considered to be composed by pure elastin.

Collagen content has been determined both gravimetrically on aortic rings as collagenase-soluble material and chemically according to the amino acid composition of the sequentially extracted pooled fragments. A hydroxyproline content of 14% (w/w) for human arterial collagen was assumed for collagen calculations after subtracting the elastin contribution from the total hydroxyproline content of the tissue (110).

The presence and the concentration of an additional polar protein component was determined chemically, either by subtracting the total collagen contribution from the amino acid composition of the sequentially extracted pooled fragments or by subtracting the elastin contribution from the amino acid composition of collagenase-purified elastin fibres (Table 10 and 11). This component was also estimated gravimetrically as trypsin- and alkali-soluble material, respectively (Table 10).

Determination of the cross-sectional area of hydrated aortic rings

In order to account for the voids within the specimens resulting from the extraction procedure, the cross-sectional area (A_o) of each hydrated ring submitted to mechanical test was expressed as the ratio between the volume of the dry sample and the midwall circumference (Cm) determined after equilibration of the sample in distilled water for 24 h at 4 °C. The volume of the dry sample was estimated according to its dry weight and the density calculated from the amino acid composition (120). The amount of hydration water was evaluated by weighing the hydrated sample after blotting it with filter paper. Cm was determined by calculating the midwall radius $Rm = (R_o + Ri)/2$, where R_o corresponds to the external radius and Ri to the internal one. R_o was obtained by measuring the external circumference (C_o) of the hydrated ring, while Ri was calculated according to the equation

$$V = L \pi (R_o^2 - Ri^2)$$

as reported by McDonald (73). The height (L) was measured on the hydrated ring, while the volume (V) was calculated by adding the volume of the dry sample to that of the hydration water.

Dynamometry

Stress-strain measurements were carried out on collagenase- and trypsin treated rings. Each hydrated ring was slung by means of specially designed metal hooks from a metal chain connected to the shaft of a multi-turn precision helipot (Beckman Instruments Ltd.) and a microdisplacement myograph transducer (Type F 10.000, E and H Instruments Co., Inc., Houston, USA). The outputs of the helipot and transducer were fed into the X and Y channels, respectively, of an XY recorder (Bryans XY/T, autoplotter series 2200, model 22020) This arrangement resulted in a direct plot of tension and variations of specimens length when the helipot shaft was rotated.

As the telationship between stress and strain was not linear, the incremental elastic modulus (E_{inc}) was calculated on the obtained stress-strain diagram at successive constant stress increments of 1.5×10^5 Nm^{-2}, with reference to the cross-sectional area ($2A_o$) of the unstretched sample, according to the equation

$$E_{inc} = \frac{\triangle\sigma}{\varepsilon_{inc}} (1 + \triangle L)$$

where $\triangle\sigma = \triangle F/2A_o$ is the stress increment over which incremental strain ε_{inc} is determined using the equation

$$\varepsilon_{inc} = \frac{L_2 - L_1}{L_1}$$

and $(1 + \triangle L) = L_1/L_o$. $2A_o$ corresponds to the cross-sectional area and L_o to the length, assumed equal to Cm/2, of the unstretched mounted sample. F is the force applied and L_1 and L_2 correspond to the length of a sample submitted to two successive stress increments. Assuming a Poisson's ratio of 0.5, L_1/L_o is the factor by which the initial cross-section ($2A_o$) has to be divided in order to account for the actual cross-section of a sample submitted to a previous stress increment (121).

Each specimen was submitted to three successive extension-relaxation cycles up to a strain of 0.3-0.4 at 0.1 mm/sec and finally extended to rupture. The stress-strain diagrams corresponding to this final extension cycle are reported in Figure 4, 5 and 8. Hysteresis was evaluated on the stress-strain diagram of the third extension-relaxation cycle. It was defined as the work not recovered upon relaxation of the sample and expressed as % of the total work performed during the extension phase. It was measured gravimetrically on the diagrams as the % ratio between the area of the hysteresis loop and that comprised between the extension curve and the strain axis.

Chemical analyses

a) Amino acid analysis

Samples for amino acid analysis were hydrolyzed in sealed glass tubes under nitrogen in constant boiling HCl (1 ml per mg of sample) at 110 °C for varying intervals (24, 36 and 72 hours) to correct for hydrolytic losses. When the hydrolysis period was over the tubes were cooled and opened. The contents were transferred to a suitable flask and taken to dryness at 40 °C on a rotary evaporator. The dried samples were stored for 18 h under reduced pressure over NaOH before analysis. Amino acid analyses were performed on both a JEOL JLC-6AH (two columns operation) and a LOCARTE (single column operation) amino acid analyzer. Standard colour values were obtained from the chromatography of suitable volumes of amino acid standard solution and of desmosine and isodesmosine solutions of known concentration. Both desmosine and isodesmosine have been expressed as lysine equivalents by adopting a molar colour coefficient corresponding to a quarter of that of desmosine and isodesmosine, respectively. Amino acids were quantitated manually from chromatograms. In Table 9 the values for threonine, serine and arginine in the amino acid composition of hot alkali purified elastin reported from reference (36) have been corrected for losses occurring during hot alkali treatment. The concentration of threonine and serine has been corrected according to Nicolet (39), while that of arginine has been modified according to the mean value $\pm$ SD of the ratio ornithine/(ornithine+arginine) found in 31 samples of hot alkali purified human aortic elastin, as reported by John and Thomas (35), and which corresponded to 0.203 ± 0.038.

When the concentration of hydroxyproline was too low for accurate estimation on a standard chromatogram, the residual hydrolyzate was analyzed for hydroxyproline according to the method of Serafini and Cessi (122).

b) Hexosamine estimations

Hydrolysis of samples for hexosamine estimations was carried out in 4 N HCl (1 ml per mg of sample) under nitrogen in sealed glass tubes at 100 °C. After 8 hours the tubes were cooled, opened and the contents transferred to a suitable flask and taken to dryness at 40 °C on a rotary evaporator. The samples were then redissolved in a suitable volume of distilled water and taken to dryness as above, the procedure being repeated twice in order to reduce the final concentration of HCl. The tubes were stored for 18 hours over NaOH pellets in an evacuated dessicator, and the total hexosamine content determined according to the procedure of Cessi and Piliego (123) after diluting the samples with distilled water. The method is based on the reaction of liberated hexosamine with acetyl acetone at pH 9.8. This gave rise to a volatile chromogen which is distilled from the reaction mixture into a solution containing p-dimethylaminobenzaldehyde to form a chromophore(absorption maximum 545 nm). This method overcomes interferences arising from chromogens formed by amino acids in the presence of neutral sugars, as such chromogens are non-volatile.

A solution containing equimolar amounts of galactosamine and glucosamine hydrochloride was used to prepare standard curves.

c) Total hexose estimations

Hexoses were quantitated using the anthrone method of Yemm and Willis (124) after hydrolysis of the samples in 1 N H_2SO_4 at 100 °C for 3 hours in sealed glass tubes under nitrogen. At the end of the hydrolysis period, the tubes were cooled, opened and the contents plus 1 N H_2SO_4 washes transferred to a volumetric flask and made up to a known volume with 1 N

H_2SO_4. A 1 ml aliquot of the diluted sample was transferred to a test tube which was immersed in a temperature controlled water bath at 4 °C and continuously shaken while 5 ml of the anthrone reagent (0.2% (w/v) of anthrone in 72% (v/v) H_2SO_4) was added dropwise from a burette. The tubes were capped and heated in a boiling water bath for 10 minutes, cooled and after 20 minutes the absorbancy was determined at 620 nm.

A solution containing equimolar amounts of glucose and galactose in 1 N H_2SO_4 was used to prepare standard curves. A protein blank was included with each determination by heating the protein with 72% (v/v) H_2SO_4 without the anthrone, in order to correct for any nonspecific colour development due to the action of the sulfuric acid.

d) Uronic acid estimations

Uronic acid was estimated using the uronic acid-carbazole reaction of Dische (125) as modified by Bitter and Muir (126). The method is based on the reaction of uronic acid with carbazole to form a chromogen which has an absorption maximum at 530 nm. Sensitivity and colour stability are increased using the modified procedure, and there is greater reproducibility of results. Optical density is a linear function of glucuronate concentration between 4 and 40 μg/ml while interference from chloride ions and oxidants is greatly reduced using this method. 5 ml of 0.025 M sodium tetraborate in concentrated sulphuric acid are added to 1 ml sample cooled continuously during shaking as for the anthrone procedure. The samples are then heated for 10 minutes in a boiling water bath, cooled to room temperature and 0.2 ml solution of carbazole, 0.125% (w/v) in ethanol, is added. The resulting solution is heated for 15 minutes in a boiling water bath, cooled and the absorbption read at 530 nm.

Standard curves were prepared by using solutions of glucuronate. A protein blank was included with each determination by heating the protein with the tetraborate-sulphuric acid reagent in order to correct for any nonspecific colour development.

Statistics

All data are presented as means ± SD unless otherwise indicated. Statistical tests include paired t-tests for comparing identical sample size and unpaired t-tests for comparing groups of different sizes. A least-square linear regression was used to calculate a regression equation correlating two variables. The correlation coefficient r was used as a measure of association and the significance level of the association was tested by comparing the computed value of r with statistical tables which give values of r corresponding to a range of probability levels for (n - 2) degrees of freedom (127). All statistics were calculated using a SR-51-II (Texas Instruments) calculator.

RESULTS

Age-related changes of the external circumference in defatted and rehydrated medial segments isolated from the upper thoracic aorta

In all the age groups the mean length of the external circumference of the proximal end of each aortic segment was longer than that of the distal end (Figure 1). The mean of the differences found between the average circumference of both ends in all the age groups corresponded to 4.3 ± 0.3 mm and was highly significant ($t = 14.333$; $P < 0.001$). Therefore, within each group the circumference of the proximal end appeared significantly greater than that of the distal end, the lowest significance level being found in the 20-29 years group ($P < 0.01$). Moreover, up to the seventh decade of life the average external circumference of both ends was positively correlated with the mean age of each group, while in the eighth decade the circumference of both ends was not significantly different from the corresponding value found in the seventh decade. When the averages of the proximal (Cp) and the distal (Cd) circumferences were expressed in mm and the mean age (A) in years, then for the first five age groups data fitted the following regressions

$$Cp = 0.626\ A + 36.2 \quad ; \quad Cd = 0.614\ A + 32.6$$

where $r = 0.9917$ for Cp and $r = 0.9963$ for Cd, while $P < 0.001$ for both. In conclusion the external circumference of the isolated medial segments progressively increased with age up to the seventh decade when its value was 48% higher than that found in the third decade.

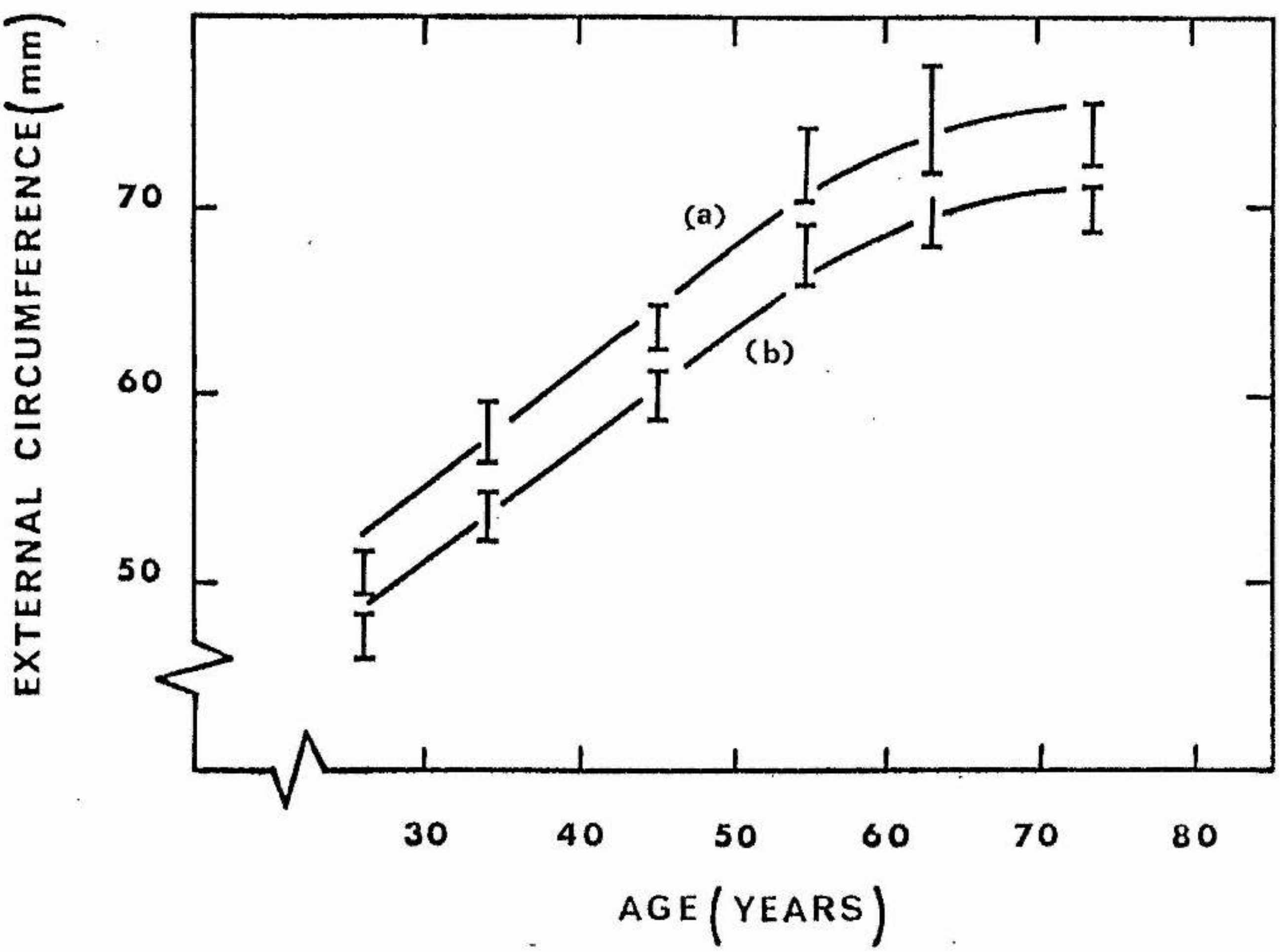


FIGURE 1

Age-related changes of the external circumference in defatted and rehydrated medial segments isolated from human upper thoracic aorta. The mean age of each group is plotted versus the average external circumfernce $\pm$ SE of the corresponding proximal (a) and distal (b) ends.

Age-related dry mass distribution within the wall of defatted and rehydrated medial aortic segments

The mean value of the dry mass concentration, expressed as dry weight (mg) per unit length (cm) in each age group of defatted and rehydrated medial segments, was positively correlated with the mean age of each age group ($r = 0.9858$; $P < 0.001$) (Figure 2). The very significant increase of the mg/cm parameter was only partly accounted for by the increase in the mineral phase and other organic components extractable by EDTA, Guan-HCl/TRIS and Guan-HCl/TRIS/DTE treatments, respectively. In fact an equally significant increase in the mg/cm parameter was found when both EDTA-extracted ($r = 0.9867$; $P < 0.001$) and Guan-HCl/TRIS plus Guan-HCl/TRIS/DTE-extracted ($r = 0.9541$; $P < 0.005$) residual segments were taken into account (Figure 2). It is evident that in the eighth decade the absolute concentration, expressed in mg/cm, of the material resistant to the sequential extraction procedure was 48% higher than that found in the third decade.

In contrast, when the data was expressed as mg/cm^2 (Figure 3), with reference to the lateral surface area of rehydrated defatted segments, the concentration of material not solubilized by the sequential extraction procedure appeared to be constant in all the age groups. In fact the regression of the mean value assumed by this parameter in all the age groups over the mean age of each group is represented by a line parallel to the age axis (Figure 3). Age-related increase of minerals and other extractable organic components accounts completely for the increase in dry mass per unit area observed before sequential extraction of rehydrated aortic media,

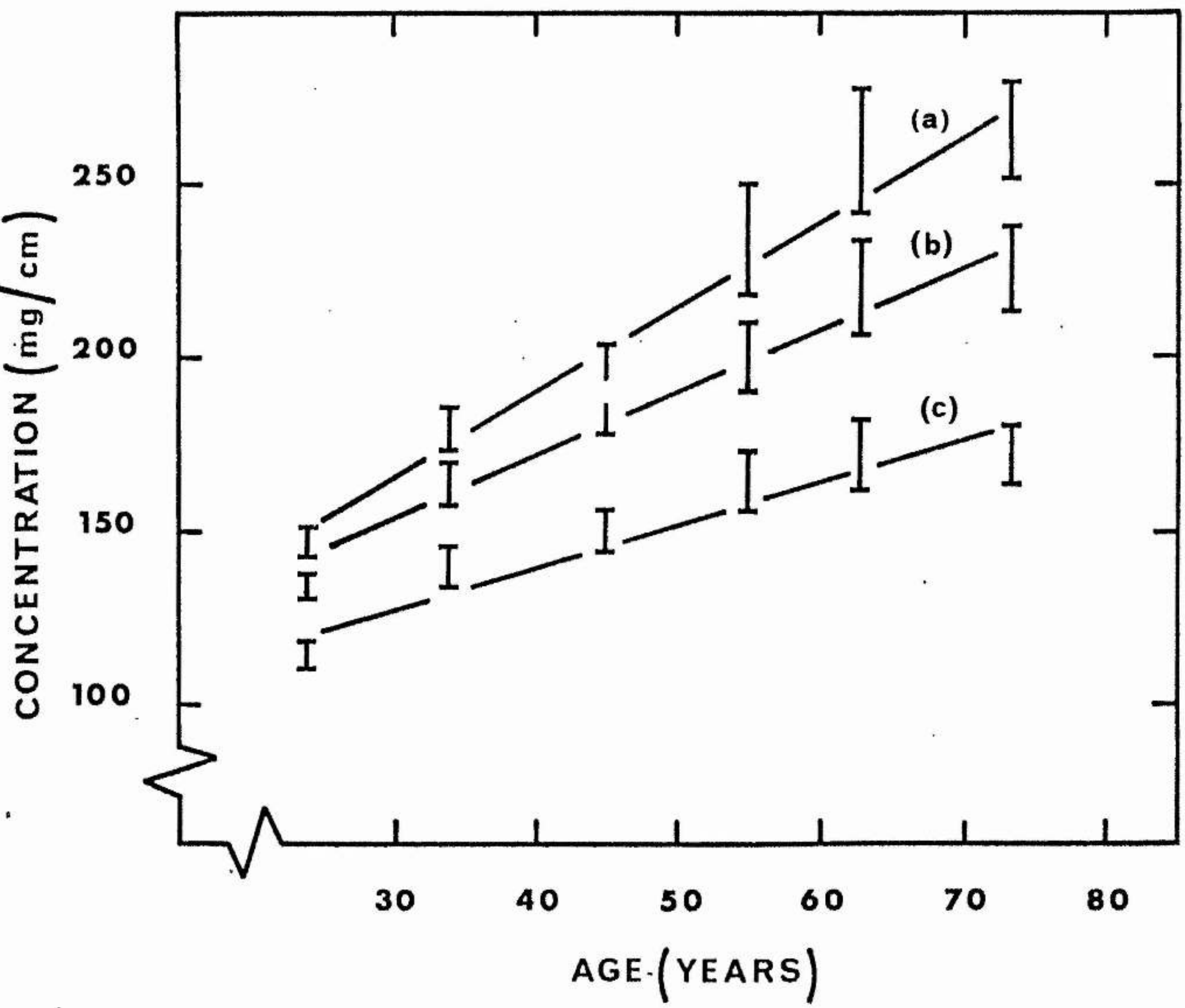


FIGURE 2

Age-related changes of the dry mass concentration in rehydrated human aortic medial segments after defatting (a), decalcification (b) and extraction with Guan-HCl/TRIS plus Guan-HCl/TRIS/DTE (c). The mean age of each group is plotted versus the average dry weight (mg) ± SE found per unit length of the rehydrated segments. The diagram represents the best fit lines calculated by least square regression analysis.

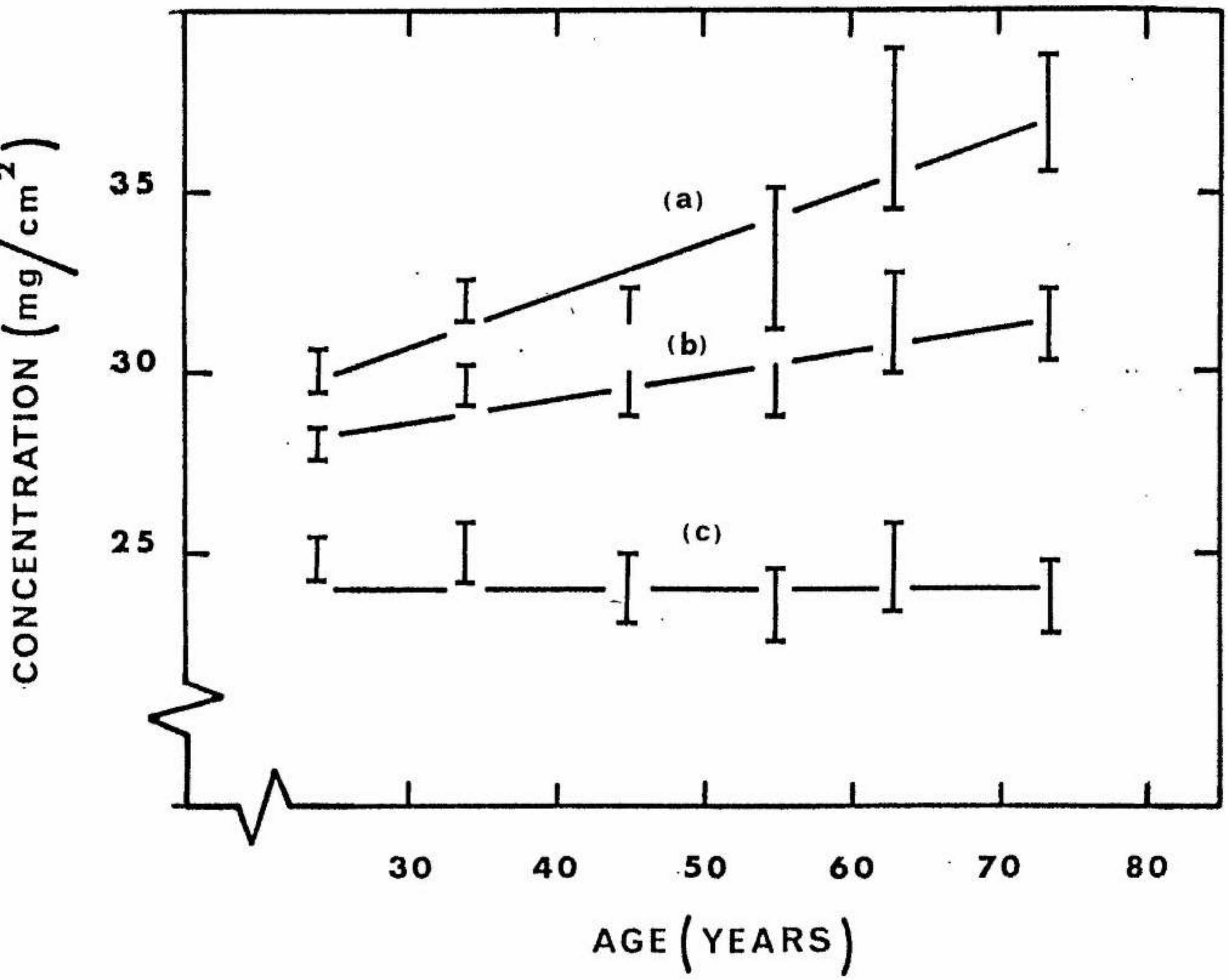


FIGURE 3

Correlation between age and dry mass concentration per unit external area (mg/cm^2) in rehydrated human aortic medial segments after defatting (a), decalcification (b) and Guan-HCl/TRIS plus Guan-HCl/TRIS/DTE extraction (c). The mean age of each group is plotted versus the average dry weight $\pm$ SE found per unit external surface area of the corresponding rehydrated segments. The diagram represents the best fit lines calculated by least-square regression analysis.

while insoluble components, namely collagen and elastin, maintain a constant distribution within the aortic media. Thus, the progressive age-related dilatation of the upper thoracic aortic segment is matched by a proportional increase in the insoluble organic components which control the mechanical resistance of the vessel.

Undialysable organic fractions solubilized by EDTA/TRIS treatment

The undialysable organic fractions of the material solubilized by EDTA/TRIS treatment consisted mainly of protein. Carbohydrate accounted for 15-24% (w/w) of total protein and carbohydrate analytically determined in the retentate, the concentration of hexoses being slightly higher than that of hexosamines (Table 3). With respect to the age of the samples, carbohydrates were more concentrated in the undiffusible fraction extracted from the youngest group, while the total amount of protein and carbohydrate was higher in the extracts from the oldest age group.

The amino acid composition of the undiffusible protein aliquot is reported in Table 4 and appears to be fairly constant, except that the methionine concentration increase with age. However, no significant linear correlation could be found between the concentration of this amino acid and the mean age of the samples from which the protein had been extracted. Polar and hydroxylated amino acids account for 50%, neutral amino acids for 44% and the aromatic ones for 6% of total amino acid residues. It was noteworthy that aspartic and glutamic acid accounted for 25%, and cysteine for 2% of all amino acid residues. The absence of desmosine and isodesmosine and the presence of a few residues of hydroxyproline indicated that a small

TABLE 3

UNDIALYSABLE FRACTIONS SOLUBILIZED BY EDTA/TRIS TREATMENT

Protein and carbohydrate composition

AGE GROUP Decade	CONCENTRATION Total protein and carbohydrate; %(w/w) of dry defatted decalcified tissue	COMPOSITION %(w/w) of total undiffusible protein and carbohydrate		
		Protein	Hexoses	Hexosamines
3	1.4	75.6	13.7	10.8
4	2.0	85.2	7.6	7.4
6	3.2	82.7	9.4	8.0
7	2.6	82.7	9.4	7.9

TABLE 4

AMINO ACID COMPOSITION OF UNDIALYSABLE FRACTIONS SOLUBILIZED BY EDTA/TRIS TREATMENT

AMINO ACID	AGE GROUP (DECADE) 3	4	6	7
OH-Lysine	0.0	0.0	0.0	0.0
Lysine	62.3	68.6	75.0	52.8
Histidine	20.9	18.4	21.9	19.1
Arginine	48.5	47.0	46.7	51.0
OH-Proline	3.0	4.8	0.9	1.6
Aspartic acid	95.4	90.6	105.2	115.0
Threonine	52.7	57.2	59.1	32.0
Serine	70.8	65.0	68.3	67.1
Glutamic acid	148.7	143.3	147.3	162.6
Proline	63.8	74.4	79.5	66.2
Glycine	83.1	86.3	80.3	79.3
Alanine	83.9	83.7	77.7	79.2
Half-cystine*	23.6	23.9	14.7	22.6
Valine	59.9	59.5	54.6	56.5
Methionine	6.2	9.5	11.7	17.6
Isoleucine	35.4	34.6	34.7	36.3
Leucine	84.3	80.7	77.7	85.4
Tyrosine	25.8	22.5	17.6	25.1
Phenylalanine	31.8	30.1	27.1	30.6
Isodesmosine	0.0	0.0	0.0	0.0
Desmosine	0.0	0.0	0.0	0.0

Values are expressed as residues/1000 amino acid residues

* Comprises cysteic acid

amount of collagen, corresponding to 0.02-0.06 (w/w) of the dry defatted decalcified tissue, had probably been extracted by this treatment, regardless of the age of the samples.

Undialysable material extracted by Guan-HCl/TRIS and Guan-HCl/TRIS/DTE treatments

Of the total material solubilized by Guan-HCl/TRIS and Guan-HCl/TRIS/DTE treatments together (determined gravimetrically, Table 11), the undiffusible protein and carbohydrate component was independent of age and corresponded to 58.4 ± 3.8 % (w/w) (expressed as the mean of the values found in all the age groups). However, with reference to the dry defatted decalcified tissue, the amount of undiffusible protein and carbohydrate solubilized by Guan-HCl/TRIS treatment was positively correlated ($r=0.8523$; $P<0.05$) with the mean age of the sample groups, while the amount of corresponding material from the Guan-HCl/TRIS/DTE extracts appeared to be constant and independent of age (Table 5). In both retentates the relative concentration of protein and single carbohydrate appeared to be constant and independent of age. In Table 5 the relative (w/w) concentration of every component has been expressed as the mean of the values found in all the age groups. With the exception of hexosamines, which are significantly ($t = 3.5823$; $P < 0.02$) more concentrated in the Guan-HCl/TRIS retentate, the relative proportion of protein and other carbohydrates was very similar in the retained fractions of both extracts.

The amino acid composition of the undiffusible fractions from five Guan-HCl/TRIS/DTE and two Guan-HCl/TRIS extracts is shown in Table 6.

TABLE 5

UNDIALYSABLE FRACTIONS SOLUBILIZED BY GUAN-HCl/TRIS AND GUAN-HCl/TRIS/DTE EXTRACTIONS

TREATMENT	CONCENTRATION Values are expressed as % (w/w) of dry, defatted and decalcified tissue							COMPOSITION Values are expressed as % (w/w) of total undiffusible protein and carbohydrate			
	Age group (decade)	3	4	5	6	7	8	Protein	Hexoses	Hexosamines	Uronic acid
Guan-HCl/TRIS		5.6	5.8	5.0	7.0	7.7	8.2	87.3 ± 1.8	5.3 ± 0.7	4.4 ± 0.9	2.9 ± 0.4
Guan-HCl/TRIS/DTE		4.8	4.4	5.2	4.7	5.5	4.4	89.0 ± 0.7	5.6 ± 1.0	2.9 ± 0.3	2.8 ± 2.8

TABLE 6

AMINO ACID COMPOSITION OF UNDIALYSABLE FRACTIONS SOLUBILIZED BY GUAN-HCl/TRIS (a) AND GUAN-HCl/TRIS/DTE (b) EXTRACTIONS

AGE GROUP (DECADE)	3	4	5	5	6	7	7
TREATMENT	(b)	(b)	(a)	(b)	(b)	(a)	(b)
AMINO ACID							
OH-Lysine	7.6	6.7	TR	9.8	7.9	TR	9.5
Lysine	42.3	49.9	54.5	42.5	39.4	52.5	46.6
Histidine	18.9	20.7	19.2	19.6	18.4	20.1	20.8
Arginine	49.8	53.3	59.4	53.0	43.1	46.3	53.8
CM-Cysteine	16.7	20.7	0.0	19.6	18.4	0.0	20.2
OH-Proline	17.1	14.3	5.8	15.0	18.8	7.9	17.6
Aspartic acid	93.5	100.0	96.4	95.0	84.9	100.4	92.3
Threonine	49.4	54.9	51.9	56.2	57.3	55.9	52.8
Serine	54.7	64.3	68.3	63.2	63.1	67.0	61.0
Glutamic acid	125.9	133.2	113.2	115.1	114.5	122.8	124.2
Proline	61.7	25.9	56.3	64.1	51.5	55.6	45.1
Glicine	123.7	121.2	102.3	116.1	136.7	95.3	127.3
Alanine	69.9	61.3	73.6	62.1	67.1	73.5	62.0
Half-cystine*	11.4	13.5	14.5	13.6	13.8	12.0	9.6
Valine	56.4	56.4	57.8	56.8	57.3	60.5	56.2
Methionine**	9.3	7.3	12.2	8.0	7.4	17.0	8.7
Isoleucine	42.4	44.1	39.1	42.6	44.6	42.6	41.1
Leucine	83.7	86.0	96.1	78.4	89.1	94.2	82.0
Tyrosine	27.9	31.8	33.9	30.1	30.1	33.6	29.6
Phenylalanine	37.5	43.3	45.4	39.2	41.6	42.9	39.6
Isodesmosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Desmosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Values are expressed as residues/1000 amino acid residues

* Comprises cysteic acid ** Comprises methionine-sulfoxide TR Traces

As the large methionine-sulfoxide peak was not completely saparated from the small hydroxyproline peak on the chromatogram, the concentration of hydroxyproline was independently determined in all the extracts by the colorimetric procedure of Serafini and Cessi (122). In the Guan-HCl/TRIS undiffusible fraction the hydroxyproline and cysteine concentration corresponded to about one half and that of methionine to about twice the corresponding value found in Guan-HCl/TRIS/DTE retentate. Moreover, in the latter case the molar ratio OH-Proline/OH-Lysine was 2.03 ± 0.34 while in the former OH-Lysine was only detected in trace amounts. With respect to total tissue hydroxyproline, 0.9 ± 0.1 % was recovered in the Guan-HCl/TRIS and 2.1 ± 0.4 % in the Guan-HCl/TRIS/DTE retentate, respectively. No age-related changes have been detected in the amino acid composition of both extracts. It is noteworthy that in the Guan-HCL/TRIS/DTE retentate only 60.8 ± 4.1 % of total cysteine residues were S-carboxymathylated under the conditions adopted for the reaction with iodoacetate. Desmosine and isodesmosine have never been detected in the undiffusible fractions, even when large amounts of hydrolyzate was analyzed.

Amino acid composition of pooled residual fragments after sequential extraction

As shown in Table 7, in pooled residual fragments obtained after sequential extraction the relative concentration of all the amino acids, with the exception of proline and histidine, could be correlated with the mean age of the corresponding group. The relationship between proline concentration and aging is probably not linear, while histidine content

TABLE 7

AMINO ACID COMPOSITION OF POOLED RESIDUES AFTER SEQUENTIAL EXTRACTION

AMINO ACID	AGE GROUP (DECADE)						P^+
	3	4	5	6	7	8	
OH-Lysine	1.6	1.2	1.7	3.0	4.1	5.2	<0.01
Lysine	13.2	13.9	13.5	17.2	18.0	18.1	<0.02
Histidine	2.2	4.7	4.4	4.8	5.1	5.0	NS
Arginine	19.2	25.9	20.8	30.4	30.0	31.0	<0.05
OH-Proline	34.4	37.1	37.4	51.7	56.3	53.5	<0.02
Aspartic acid	15.5	19.6	28.3	30.1	32.0	29.9	<0.02
Threonine	10.4	12.5	18.8	21.4	20.0	18.9	<0.05
Serine	13.9	17.9	22.6	22.2	29.0	26.7	<0.01
Glutamic acid	33.3	39.9	48.8	53.2	57.1	55.6	<0.005
Proline	120.0	123.8	129.5	113.7	109.2	108.3	NS
Glycine	347.8	334.0	290.1	269.8	270.2	268.8	<0.01
Alanine	188.2	178.7	170.7	161.0	150.2	155.9	<0.001
Half-cystine§	3.3	3.5	6.8	8.4	9.2	9.4	<0.005
Valine	96.0	88.5	88.3	82.1	72.5	79.3	<0.02
Methionine§§	2.3	3.1	5.8	6.3	7.2	7.3	<0.005
Isoleucine	17.4	16.0	21.2	24.1	23.9	24.1	<0.02
Leucine	44.0	41.5	48.0	52.5	51.2	52.1	<0.05
Tyrosine	12.4	11.8	16.8	19.3	18.7	19.9	<0.01
Phenylalanine	16.4	14.7	17.7	23.0	22.4	22.9	<0.02
Isodesmosine*	4.4	3.8	3.6	3.2	3.0	3.0	<0.005
Desmosine*	6.8	6.2	5.0	5.5	4.9	5.1	<0.05
Lysinonorleucine*	1.2	1.0	ND	ND	ND	ND	ND

Values are expressed as residues/1000 amino acid residues

+ Significance level of the correlation between age and amino acid concentration; NS P>0.05.

§ Comprises cysteic acid   §§ Comprises methionine sulfoxide

* Expressed as Lysine equivalents

does not show any remarkable age-related change. The significance of the association between age and amino acid composition expressed in residues/ 1000 residues has been tested by comparing the calculated value of r with the tabulated values of r corresponding to a range of probability levels, for (n - 2) degrees of freedom (127). The proportion of aromatic, polar and hydroxylated amino acids, leucine, isolecine and half-cystine increase significantly with age, while that of the other neutral amino acids, desmosine and isodesmosine decrease significantly.

When the lowest and the highest age groups are compared it is seen that the concentration of hydroxyproline nearly doubles, while that of desmosine plus isodesmosine decreases by about 30 %. On the other hand, half-cystine content triples while that of all the polar amino acids doubles. According to the amino acid composition reported in Table 7, the total amount of collagen and elastin, calculated as previously outlined, does not account for the total amino acid recovery. With increasing age an increasing amount of an additional protein component associates with the insoluble collagen and elastin. The concentration of this additional component has been calculated by difference and reported in Table 11 together with that of collagen and elastin.

Amino acid composition and gravimetric changes in the residual aortic rings after treatment with collagenase and trypsin

As shown in Table 8, the amino acid composition of the insoluble residue obtained after collagenase treatment corresponds to the general pattern expected for human aortic elastin. There is a high content of

TABLE 8

AMINO ACID COMPOSITION OF COLLAGENASE-TREATED UPPER THORACIC PROXIMAL (Prox) AND DISTAL (Dist) AORTIC RINGS

RING	Prox	Dist	Prox	Dist	Prox	Dist	Prox	Dist	Prox	Dist	Dist	P*
AGE (YEARS)	19	29	39		44		56		65		76	
AMINO ACID												
OH-Lysine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND	0.0	
Lysine	5.4	7.7	10.1	8.1	11.5	11.7	13.2	10.3	12.7	13.3	16.6	<0.001
Histidine	0.8	1.1	1.1	1.5	2.8	2.7	3.0	2.5	3.2	3.7	4.5	<0.001
Arginine	9.3	12.1	11.9	12.8	13.8	14.7	16.4	15.7	17.1	20.0	22.1	<0.001
OH-Proline	6.9	7.7	9.0	7.4	7.5	6.3	11.1	12.2	11.2	14.5	12.0	<0.005
Aspartic acid	7.9	12.5	16.4	12.8	16.8	19.3	22.1	18.9	22.4	20.7	24.9	<0.001
Threonine	16.1	17.5	19.6	18.4	23.3	21.6	20.9	20.4	27.2	22.2	26.1	<0.001
Serine	13.6	19.4	23.1	18.5	20.3	23.0	27.2	22.1	24.2	26.1	25.0	<0.005
Glutamic acid	21.4	30.7	33.9	28.8	32.1	35.9	42.0	36.8	38.9	43.4	42.2	=0.001
Proline	129.0	119.8	116.3	118.8	114.3	116.7	113.2	116.7	113.7	114.8	110.2	<0.001
Glycine	293.7	279.6	275.5	282.7	263.0	271.6	263.5	276.0	259.2	273.8	253.5	<0.005
Alanine	228.0	217.0	213.6	220.7	213.5	206.6	196.9	202.3	200.8	190.0	189.2	<0.001
Valine	123.8	119.6	119.4	125.1	118.7	120.7	114.3	115.4	113.7	108.4	108.2	<0.001
Methionine§	0.0	1.2	2.7	2.2	2.6	3.4	3.6	4.1	3.6	4.9	4.5	<0.001
Isoleucine	24.5	29.0	27.0	25.8	29.1	27.2	27.6	26.4	28.0	26.1	29.8	NS
Leucine	59.1	61.7	63.1	61.3	66.7	63.0	64.4	64.3	63.3	59.2	65.9	NS
Tyrosine	19.7	21.0	19.9	18.2	21.8	20.6	22.8	21.1	22.0	23.3	24.5	<0.005
Phenylalanine	23.9	28.6	24.5	21.7	27.7	22.8	25.7	22.3	26.8	22.4	28.7	NS
Isodesmosine**	6.0	6.2	5.1	5.2	5.5	4.6	4.4	4.6	5.1	4.0	4.4	<0.005
Desmosine**	8.9	7.7	7.0	8.4	8.1	7.7	6.6	7.1	6.4	5.8	6.6	
Lysinonorleucine**	1.8	ND	1.0	1.4	0.9	ND	0.9	1.0	0.6	1.1	1.2	NS

Values are expressed as residues/1000 amino acid residues

* Significance level of the correlation between amino acid concentration and age; NS P>0.05
§ Comprises methionine-sulfoxide
** Expressed as lysine equivalents

non-polar amino acids, especially glycine, a low hydroxyproline and a high desmosine plus isodesmosine concentration. However, with the exception of isoleucine, leucine and phenylalanine, all the amino acids vary in concentration and show a highly significant correlation with the age of the subject from which the elastin has been isolated. The mean values calculated for the concentration of leucine, isoleucine and phenylalanine (Table 8) corresponded to 62.9 ± 2.4, 27.3 ± 1.6 and 25.0 ± 2.6 residues per 1000 total amino acid residues, respectively. On the contrary the concentration of both neutral amino acids and desmosine plus isodesmosine decreased with increasing age, while that of polar amino acids and of hydroxyproline showed the opposite trend. It evident that with increasing age collagenase-treated elastin fibres are progressively contaminated by a desmosine-free polar protein component. The amount of this additional component in each sample has been determined by subtracting the aliquot of pure elastin from the total amino acid recovery as reported in Table 10. As previously mentioned, elastin concentration was calculated on the basis of desmosine plus isodesmosine content, with reference to the average amino acid composition of trypsin-purified elastin fibres as reported in Table 9. From these data, it is evident that trypsin treatment almost completely solubilizes the additional polar protein component, since in the insoluble residue the concentration of both non-polar amino acids and desmosines was higher than that found in collagenase-treated samples and was independent of age. However, minor age-related variations are detectable among some polar amino acids and glycine. In fact, the

TABLE 9

AMINO ACID COMPOSITION OF ELASTINS ISOLATED FROM HUMAN THORACIC AORTA

METHOD OF PURIFICATION	COLLAGENASE + TRYPSIN							AUTOCLAVING + TRYPSIN + CNBr + β-MERCAPTOETHANOL IN 6 M UREA*	EDTA + NaOH**
AGE (YEARS)	29	39	44	56	65	76		32	
AMINO ACID							Mean ± SD		Mean ± SD
Hydroxylysine	0.0	0.0	0.0	0.0	0.0	0.0	0.0±0.0	ND	ND
Lysine	2.4	3.5	3.7	3.9	5.8	3.9	3.9±1.1	4	6.5±0.6
Histidine	0.0	0.0	0.0	0.0	0.0	0.0	0.0±0.0	0.5	0.7±0.1
Arginine	6.7	6.7	6.5	6.5	8.5	8.3	7.2±0.9	9	(6.4) 7.8±0.8
OH-Proline	6.0	6.6	6.7	6.2	6.7	6.7	6.5±0.3	10	7.5±0.7
Aspartic acid	5.3	5.1	4.9	5.3	7.9	9.3	6.3±1.8	6	6.7±0.7
Threonine	11.0	12.2	15.0	12.8	15.0	16.0	13.7±2.0	12	(7.5) 13.9±0.7
Serine	8.6	8.5	9.3	9.0	11.0	9.7	9.3±0.9	8	(7.9) 11.5±0.9
Glutamic acid	15.9	18.8	19.4	18.0	21.3	23.5	19.5±2.6	18	20.6±1.1
Proline	127.2	128.7	142.3	133.8	122.4	121.9	129.4±7.7	129	115.4±1.9
Glycine	297.5	295.2	298.2	294.5	283.6	283.6	292.1±6.7	291	296.2±3.1
Alanine	232.6	229.2	223.3	231.3	227.4	227.0	228.5±3.3	230	242.0±2.5
Valine	141.1	143.3	133.5	141.5	147.3	154.2	143.5±6.9	141	125.2±2.8
Methionine	0.0	0.0	0.0	0.0	0.0	0.0	0.0±0.0	0	0.4±0.2
Isoleucine	23.6	24.3	23.8	23.4	24.1	21.7	23.5±0.9	23	24.2±1.1
Leucine	60.9	56.2	54.1	54.4	58.0	53.0	56.1±2.9	57	57.0±2.3
Tyrosine	22.7	22.9	22.5	22.6	23.9	23.3	23.0±0.5	23	24.6±1.3
Phenylalanine	21.9	20.4	20.9	20.4	19.4	20.3	20.5±0.8	22	22.9±0.7
Isodesmosine§	6.3	7.3	6.9	7.0	7.7	7.5	7.1±0.5	8.7	7.7±0.1
Desmosine§	10.4	11.3	9.0	9.4	10.1	10.3	10.1±0.8	11.0	9.0±0.0

Values are expressed as residues/1000 amino acid residues
* Modified from reference (23) § Expressed as lysine equivalents
** Modified from reference (36): Mean ± SD calculated on three different samples,as specified in Results(p.50); original uncorrected values are given in parenthesis

concentration of aspartic acid, threonine and glutamic acid increased slightly but significantly with age ($P < 0.05$, $P < 0.05$ and $P < 0.02$, respectively), while that of glycine showed a proportional decrease ($P < 0.05$). Nevertheless, the size of these variations was rather small as in the 76 year old sample the total content of aspartic acid, threonine and glutamic acid corresponds to 49 residues, while in the 29 year old sample these three amino acids accounted for 32 residues per 1000 total amino acid residues. Accordingly, in Table 9 the concentration of each amino acid has also been reported as the mean $\pm$ SD of the values determined in all the trypsin-purified Interm rings isolated from subjects of different ages and compared with the amino acid composition of human aortic elastin purified with both the method of Starcher and Galione (23) and EDTA plus hot alkali (36). Virtually no compositional differences were detectable with the elastin purified by the method of Starcher and Galione (23), while some significant differences could be found if the comparison was made with the average amino acid composition of three different samples of human aortic elastin purified with EDTA plus hot alkali and isolated from young normal aorta, plaque areas and non-plaque areas of old aortae, respectively (36). If threonine, serine and arginine values are corrected for losses occurring during hot alkali treatment (39), the concentration of polar amino and hydroxylated amino acids is very similar in both the enzyme and hot alkali preparations, while significant differences are present among proline, alanine and valine concentration. Assuming that population variances are not equal, because the samples have been drawn from independent populations, it could be demonstrated that proline ($t = 4.1898$; $P < 0.01$), and valine ($t = 5.6146$; $P < 0.001$) are significantly more concentrated in enzymically purified elastin, while alanine ($t = 6.8871$; $P < 0.001$) is more concentrated in elastin fibres purified with EDTA plus hot alkali.

Gravimetric changes in the residual aortic rings after treatment with alkali

The amount (w/w) of material solubilized with trypsin and hot alkali treatment, and that of the additional polar protein component, determined on the basis of the amino acid composition of the collagenase-treated rings, have been reported in Table 10. The concentration of trypsin-soluble (column A), alkali-soluble (column B) material and additional polar protein component (column C) were positively and highly significantly correlated with the age of the corresponding sample: $P < 0.001$ for the data tabulated under column A, B and C. Moreover, the amount of material solubilized with hot alkali did not differ significantly ($t = 0.3929$) from the amount of chemically determined additional polar protein and no significant difference could be found between the amount of trypsin-soluble material (column A) and the mean of the values of each segment tabulated under column B ($t = 1.0855$) or column C ($t = 0.1066$).

Age-related concentration (relative and absolute) of connective tissue matrix components within the media of defatted human aortic segments

The relative and absolute concentration of connective tissue matrix components in the media of dry defatted and decalcified (% w/w) and defatted

TABLE 10

CONCENTRATION IN THE COLLAGENASE-TREATED RINGS OF (A)TRYPSIN-SOLUBLE MATERIAL, (B) ALKALI-SOLUBLE MATERIAL, (C) ADDITIONAL INSOLUBLE PROTEIN COMPONENT

AGE (YEARS)	RING	A*	B*	C**
29	INTERM	17.5		
29	DISTAL		19.3	19.9
39	PROXIMAL		26.2	36.9
39	INTERM	28.2		
39	DISTAL		27.1	28.5
44	PROXIMAL		29.8	19.0
44	INTERM	27.2		
44	DISTAL		31.1	26.0
56	PROXIMAL		41.5	36.0
56	INTERM	34.1		
56	DISTAL		37.9	31.6
65	PROXIMAL		30.4	37.9
65	INTERM	38.5		
65	DISTAL		43.2	47.3
76	INTERM	46.8		
76	DISTAL		47.0	41.9

Values are expressed as % (w/w) of dry weight

* Gravimetric difference of residues before and after treatment in caption

** From compositional data in Table 4 (cf. Results, p. 47) rehydrated segments (mg/cm), has been reported in Table 11. The concentrations of Guan-HCl/TRIS and Guan-HCL/TRIS/DTE soluble material were determined gravimetrically on both groups and on single labelled segments of known age, while the concentrations of collagen, elastin and desmosine-free polar protein component were determined both chemically, from the amino acid composition of pooled residual fragments, and gravimetrically on aortic rings isolated from single labelled segments. In the latter case, collagen concentration value corresponded to the average amount of collagenase-soluble material found in Proximal, Interm and Distal rings, while that of elastin was calculated from the proportion of trypsin-soluble and insoluble material in Interm rings.

The absolute concentration of elastin was independent of age and corresponded to a mean value of 70.5 $\pm$ 5.1 mg/cm, while its relative concentration was negatively and significantly correlated with age. Although the absolute concentration of elastin has been determined in single and pooled samples by different procedures (gravimetric and chemical, respectively) the scattering of the data was reasonably low, since for a $P < 0.05$ the confidence limits of the average concentration corresponded to the range $\pm$ 3.2%. In the oldest samples the relative concentration of elastin decreased by about 50% with respect to the youngest ones. On the contrary, the relative concentration of both collagen and total material solubilized by Guan-HCl/TRIS plus Guan-HCL/TRIS/DTE treatments were positively and significantly correlated with age and increased by about 30% in the oldest samples, while their absolute

TABLE 11

RELATIVE AND ABSOLUTE CONCENTRATION OF SOLUBLE AND INSOLUBLE COMPONENT OF THE TUNICA MEDIA OF HUMAN UPPER THORACIC AORTA

SAMPLE AGE (YEARS)		Pool 20-29	Single 29	Pool 30-39	Single 39	Pool 40-49	Single 44	Pool 50-59	Single 56	Pool 60-69	Single 65	Pool 70-79	Single 76	P*
COMPONENT SOLUBLE IN GUAN-HCl/TRIS+ GUAN-HCl/TRIS/DTE	%(w/w)	$17.1^{\dotplus 1}$	$15.6^{\mp 1}$	17.2	22.0	19.9	18.4	19.8	23.4	21.2	20.4	21.8	19.5	<0.05
	mg/cm	$23.6^{\dotplus 1}$	$24.0^{\mp 1}$	28.2	37.6	36.9	33.0	39.7	47.2	46.8	44.5	49.2	46.5	<0.001
ELASTIN	%(w/w)	$53.1^{\dotplus 2}$	$49.1^{\mp 2}$	46.3	37.9	37.7	40.4	37.1	30.7	32.5	29.6	33.7	27.3	<0.001
	mg/cm	$73.4^{\dotplus 2}$	$75.5^{\mp 2}$	75.8	64.7	69.9	72.4	74.4	62.0	71.7	64.6	76.0	65.1	NS
COLLAGEN	%(w/w)	$23.5^{\dotplus 3}$	$24.9^{\mp 3}$	25.6	25.2	25.0	26.1	33.9	30.0	36.7	31.5	34.3	29.1	<0.005
	mg/cm	$32.5^{\dotplus 3}$	$38.3^{\mp 3}$	41.9	43.0	46.3	46.7	68.0	60.5	81.0	68.8	77.3	69.4	<0.001
ADDITIONAL IN-SOLUBLE PROTEIN COMPONENT	%(w/w)	$6.3^{\dotplus 4}$	$10.4^{\mp 4}$	10.9	14.9	17.4	15.1	9.2	15.9	9.6	18.5	10.2	24.1	NS
	mg/cm	$8.7^{\dotplus 4}$	$16.0^{\mp 4}$	17.8	25.4	32.2	27.0	18.5	32.1	21.2	40.4	23.0	57.5	<0.02

Values are expressed as: (a) %(w/w) with reference to dry, defatted and decalcified tissue weight and (b) mg/cm with reference to the length of the defatted and rehydrated segments.

* Significance level of the correlation between age and concentration values. NS = $P > 0.05$

$\dotplus$ Pool $\mp$ Single : these are inserted only in the first two columns but apply in the same order throughout.

$\dotplus$1 and $\mp$1 : gravimetric difference of residues before and after treatment with chaotropic solutions

$\dotplus$2 : from desmosine+isodesmosine concentration $\mp$2 : weight of residue after all treatments

$\dotplus$3 : from($HOPro_{total}$-$HOPro_{elastin}$) concentration $\mp$3 : gravimetric difference before and after collagenase treatment

$\dotplus$4 : total protein - (collagen + elastin) before collagenase treatment

$\mp$4 : total protein - elastin after collagenase treatment concentration almost doubled. The relative concentration of the desmosine-free polar protein component was not significantly correlated with age, its mean value corresponding to 13.5 $\pm$ 5.0 % (w/w). The resulting high SD value was due mainly to the great difference found between the gravimetric and the chemical valuesof this component in the oldest samples. However, in spite of the great scatter of the data, the absolute concentration of the desmosine-free polar protein component appeared to be positively and significantly correlated with age.

Mechanical properties of aortic rings after treatment with collagenase and trypsin

The stress-strain diagrams produced during the fourth extension cycle by the Proximal and Distal collagenase-treated rings have been reported in Figure 4 and 5, respectively. At low strain values the relationship between stress and strain was not linear and showed an exponential shape up to a strain value of 0.30. However, in the range of 0.45-0.55 for Proximal and of 0.30-0.60 for Distal rings the diagrams became linear. With increasing age both flex point and rupture of the samples occurred at progressively lower strain values, while the slope of the linear part of the diagram became steeper. The mean value for the breaking stress of the six collagenase-treated (Proximal and Distal) rings isolated from the 29, 39 and 39 year old aortae was 2.02 $\pm$ 0.06 x 10^6 Nm^{-2}, while the corresponding value for the six rings (Proximal and Distal) isolated from the oldest aortae was 1.80 $\pm$ 0.31 x 10^6 Nm^{-2}. In Figure 6 and 7 the values of the incremental modulus (E_{inc}) have been

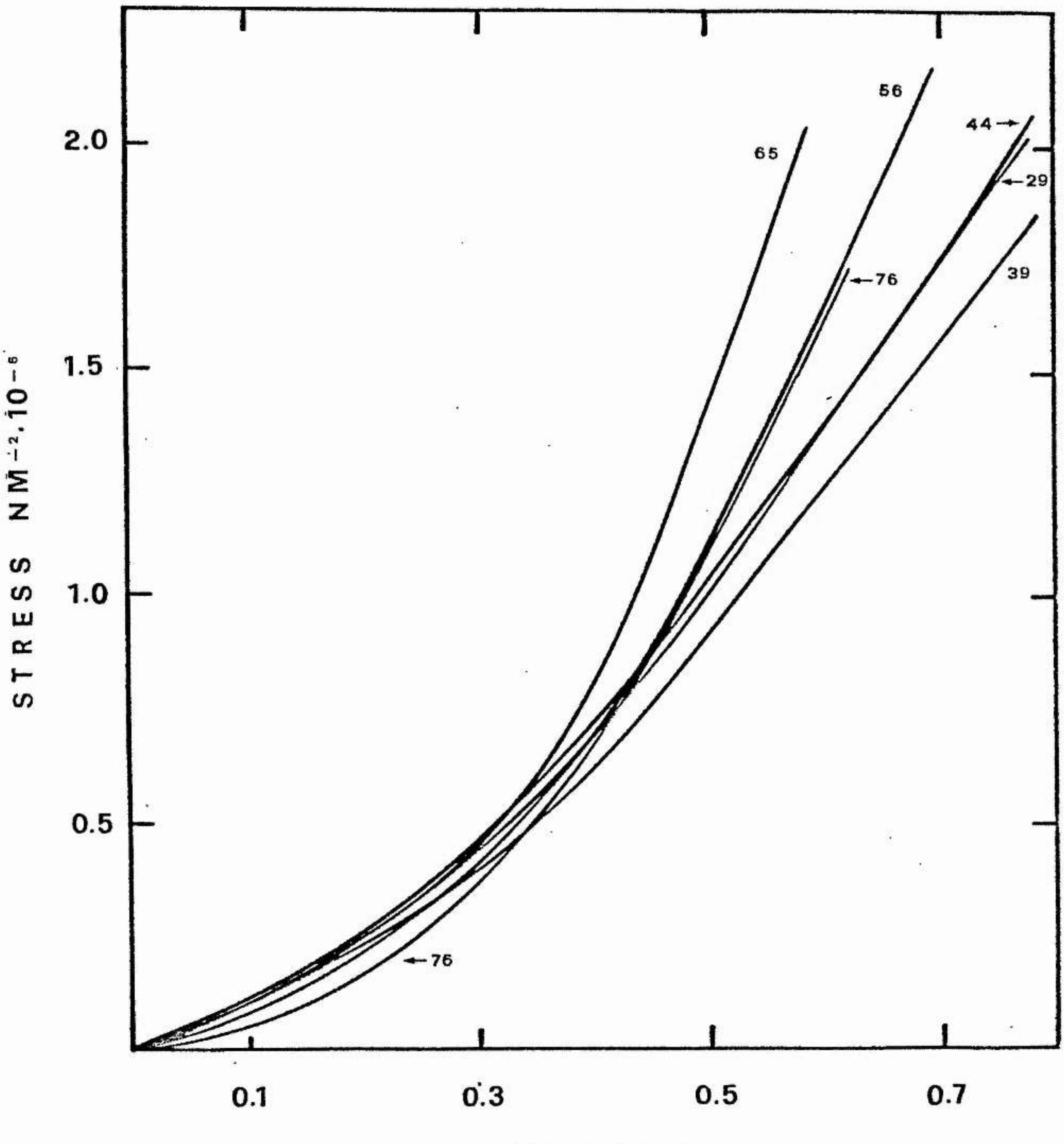


FIGURE 4

Stress-strain diagram of collagenase-treated aortic rings from proximal end of upper thoracic aortae. The age (years) of each sample is given.

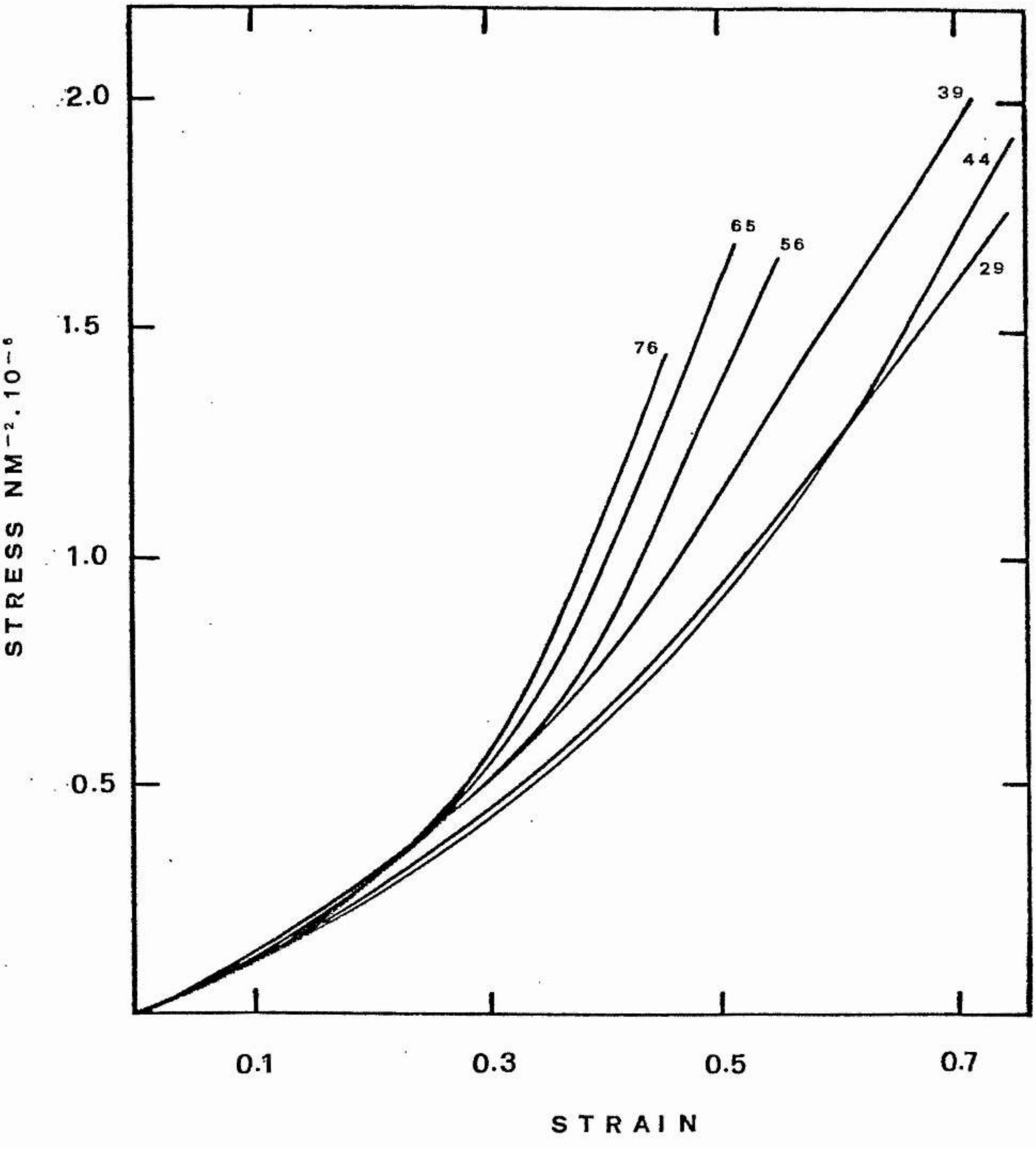


FIGURE 5

Stress-strain diagram of collagenase-treated aortic rings from distal end of upper thoracic aortae. The age (years) of each sample is given.

plotted versus stress values. The relationship between E_{inc} and stress became linear for all the samples at a stress value around 1.4×10^6 Nm^{-2}, while the curves were almost parallel at higher stress values. The average E_{inc} calculated at this stress value for the six youngest samples was $8.83 \pm 1.03 \times 10^6$ Nm^{-2}, while that for the six oldest rings corresponded to $12.2 \pm 1.5 \times 10^6$ Nm^{-2}. Assuming that population variances were equal, the difference between these two mean values was found to be significant ($t = 3.5520$; $P<0.01$). Moreover, at this stress value, the single E_{inc} values were significantly correlated ($P<0.01$) with sample age and the relationship could fit the following regression

$$E_{inc} = 0.095\ A + 5.59$$

where E_{inc} is expressed as 10^6 Nm^{-2} and age (A) as years. The stress-strain diagrams produced by the trypsin-treated Interm rings and the corresponding plot of E_{inc} versus stress have been reported in Figure 8. All the tested samples showed the same mechanical behaviour and produced non-linear superimposable diagrams, with the exception that the 76 year old sample showed a mechanical failure at a strain of 0.30 and then deviated from the pattern shown by the other samples. The breaking strain of the 29 year old sample corresponded to 0.74 while the corresponding average value for the 39, 44 and 56 year old samples was 0.50 ± 0.05 (the 65 year old sample was damaged during preparation). The breaking stress of the 29 year old sample was 1.20×10^6 Nm^{-2}, while that of the three older samples corresponded to $0.66 \pm 0.07 \times 10^6$ Nm^{-2}. The E_{inc} diagram shown in Figure 8 has also been reported in Figure 6 and 7 for comparison purposes. At corresponding stress

FIGURE 6

Incremental modulus (E_{inc}) of collagenase-treated aortic rings from the proximal end of upper thoracic aorta.

□ = 29 years

▲ = 39 years

● = 44 years

○ = 56 years

■ = 65 years

△ = 76 years

(The incremental modulus of collagenase- and trypsin-treated Interm rings is given for comparison, — ◑ —)

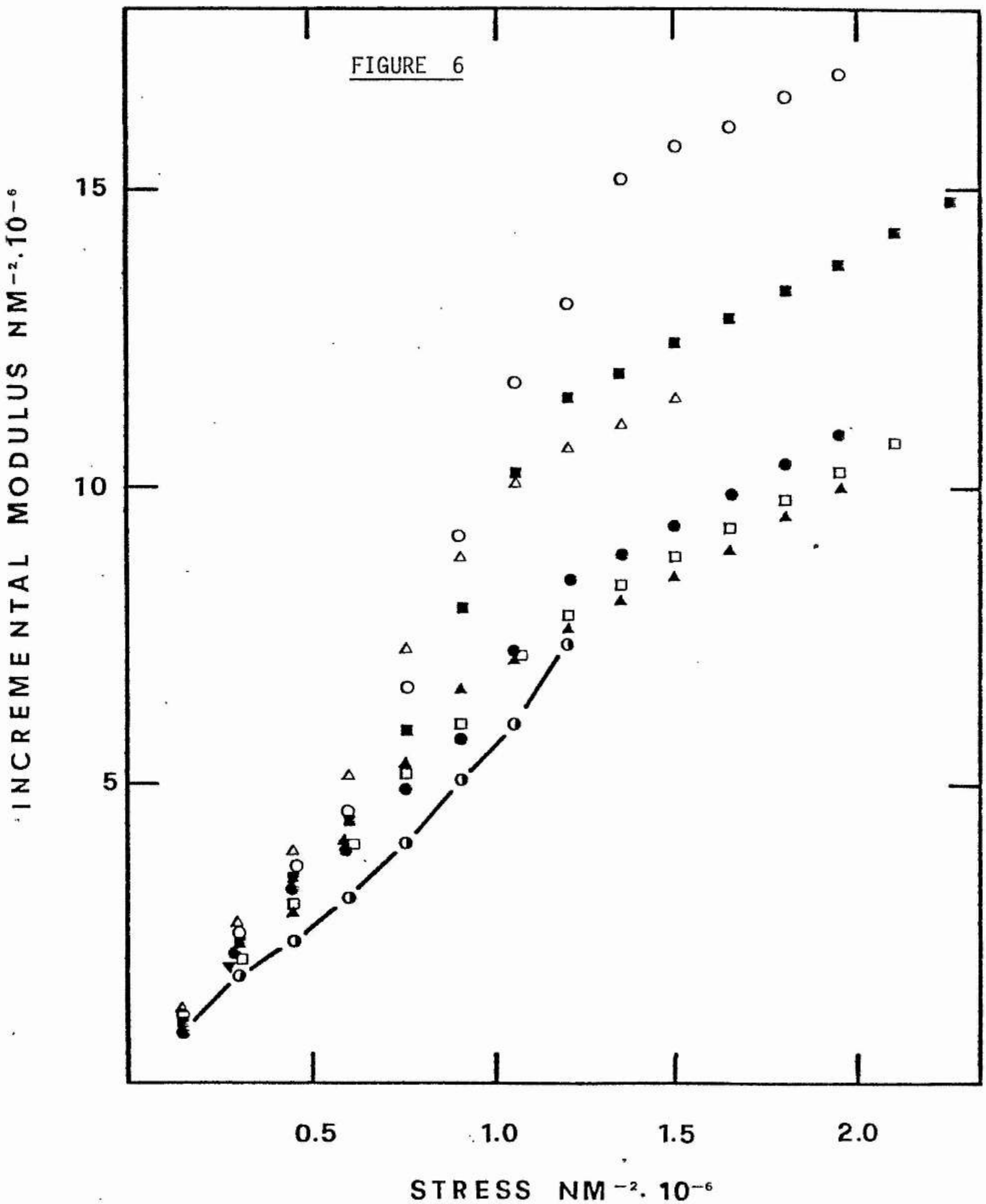


FIGURE 6
INCREMENTAL MODULUS NM⁻².10⁻⁶
15
10
5
0.5
1.0
1.5
2.0
STRESS NM⁻².10⁻⁶

FIGURE 7

Incremental modulus (E_{inc}) of collagenase-treated aortic rings from the distal end of upper thoracic aorta.

□ = 29 years

▲ = 39 years

● = 44 years

○ = 56 years

■ = 65 years

△ = 76 years

(The incremental modulus of collagenase- and trypsin treated Interm rings is given for comparison, —◐—)

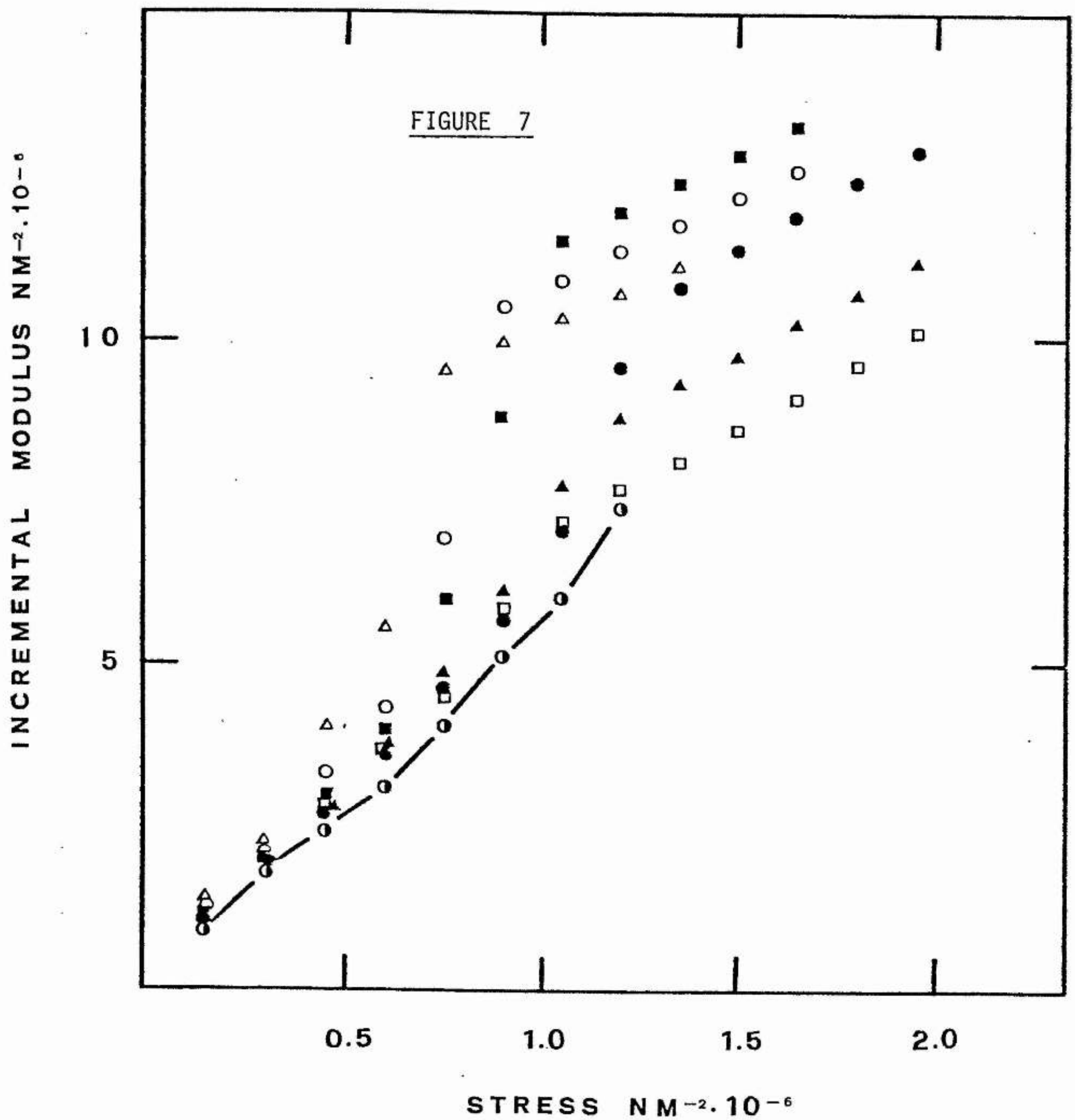


FIGURE 7
INCREMENTAL MODULUS NM⁻².10⁻⁸
10
5
0.5
1.0
1.5
2.0
STRESS NM⁻².10⁻⁶

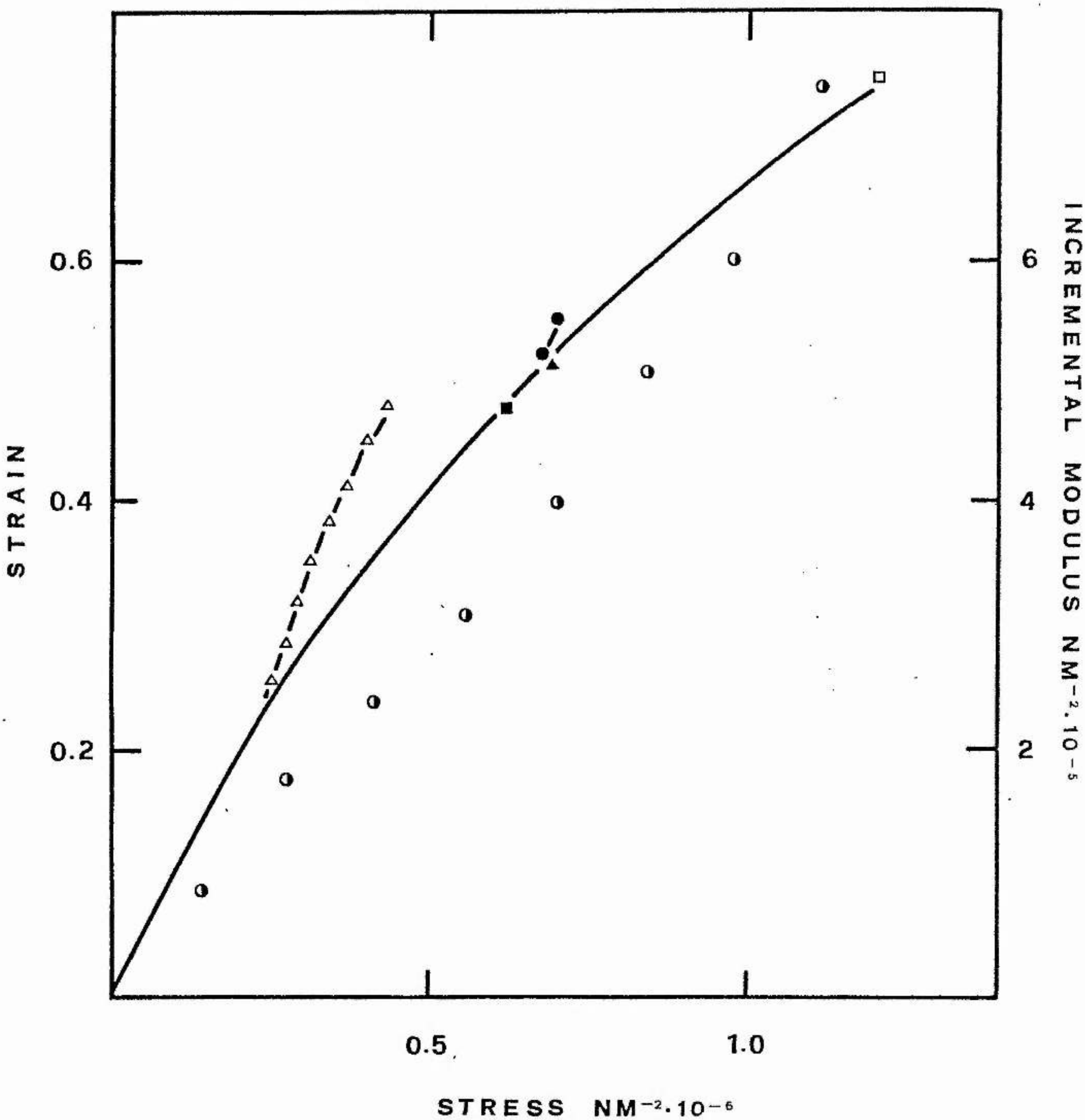


FIGURE 8

Stress-strain curve and incremental modulus of collagenase- and trypsin-treated aortic Interm rings. —□— = 29 years; —▲— = 39 years; —●— = 44 years; —■— = 56 years; —△— = 76 years; ◑ = incremental modulus.

values the E_{inc} of trypsin-treated rings was lower but very similar to that shown by the youngest collagenase-treated rings in the non-linear part of the stress-strain diagram.

In order to illustrate the elastic hysteresis shown by the aortic rings during stress-strain experiments, two diagrams produced during the third extension-relaxation cycle by a young and old sample have been reported in Figure 9. The hysteresis values found in all the samples were plotted versus the corresponding sample age and reported in Figure 10. In the collagenase-treated rings the amount of work not recovered during the relaxation phase was positively and significantly correlated with age ($P<0.001$) and with the concentration of the alkali-soluble material found in corresponding samples ($P<0.005$) as shown in Table 10. In the trypsin-treated rings hysteresis values did not show significant age-related changes and corresponded to a mean value of 11.05 ± 1.13 %.

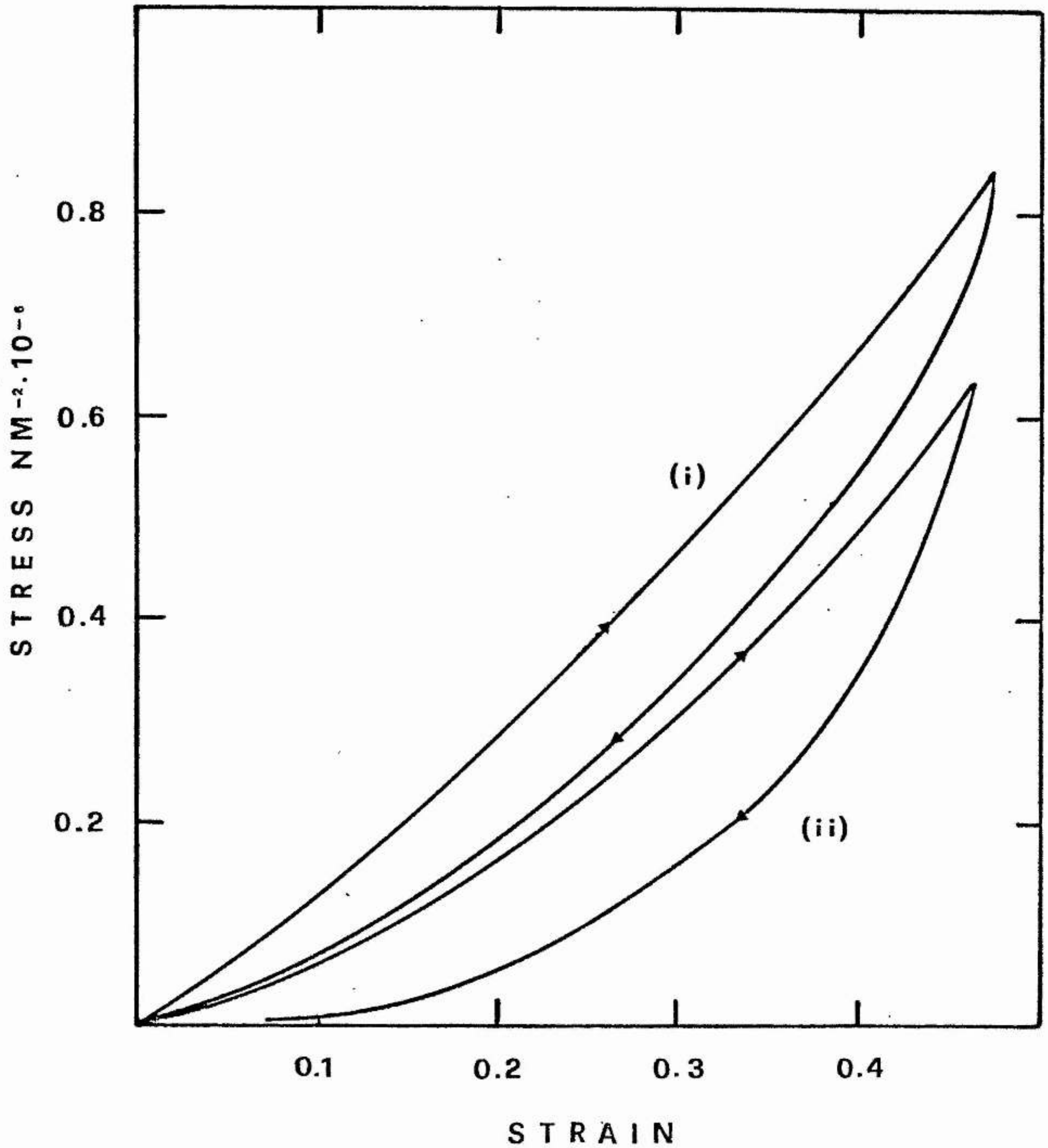


FIGURE 9

Extension-relaxation curves of collagenase-treated aortic rings from the proximal end of upper thoracic aorta. (i) aged 29 years; (ii) aged 76 years.

FIGURE 10

Changes in hysteresis with age of Proximal, Interm and Distal aortic rings.

■ = collagenase-treated Proximal rings

● = collagenase-treated Distal rings

▲ = collagenase- and trypsin-treated Interm rings

The diagram represents the best fit lines calculated by least square regression analysis.

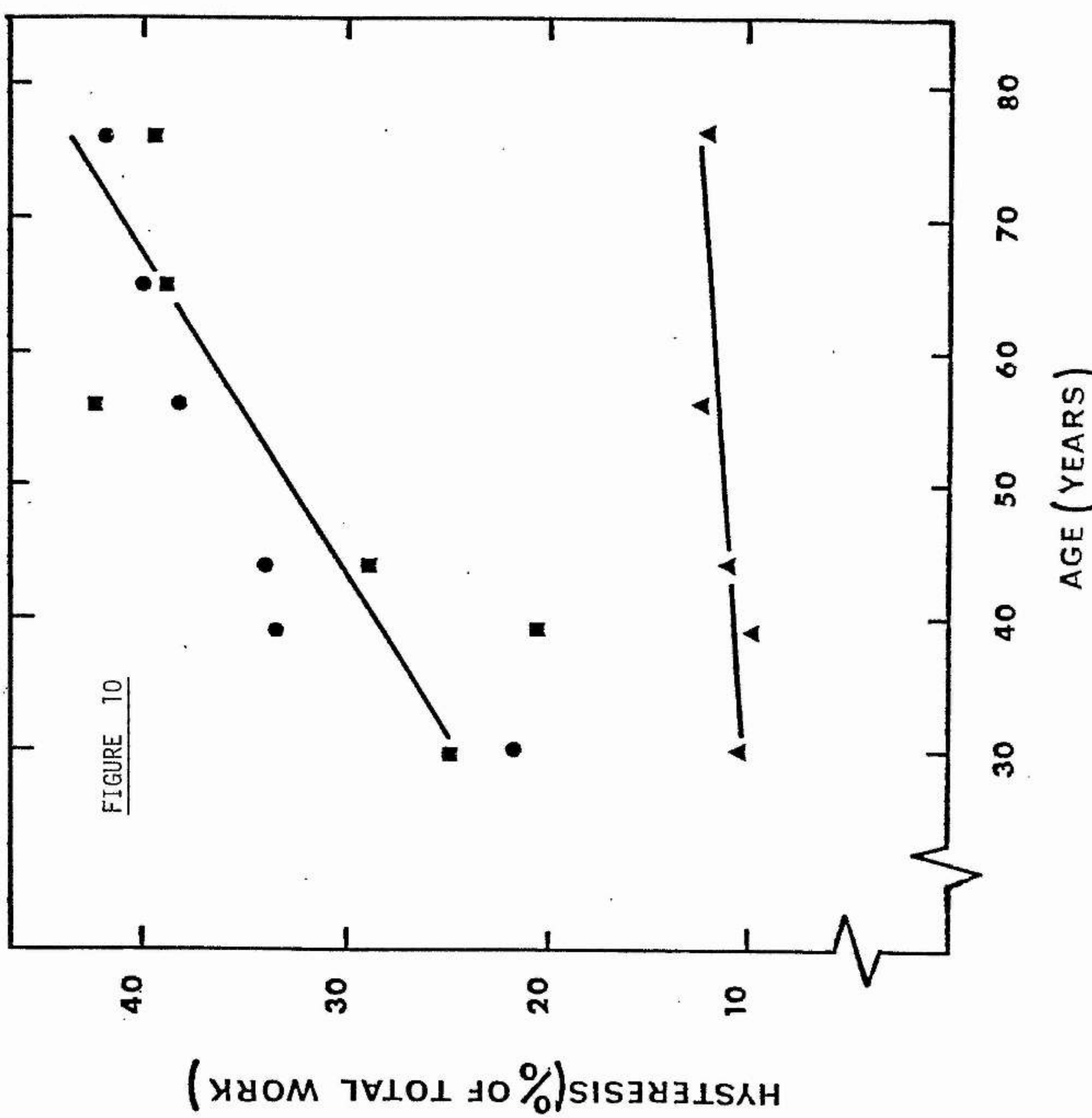


FIGURE 10

DISCUSSION

Several investigators have proved that both the circumference and the amount of tissue composing the media of the human aorta gradually increase with advancing age (3, 108, 117). The present study has led to the same conclusion. The age-related radial dilatation of the vessel paralleled an increase in the dry mass per unit area (of the wall) and was associated with a considerable rearrangement of all matrix constituents. These changes were related to the increasing concentration of both the mineral phase and the organic fractions solubilized in denaturing and reducing conditions while the relative concentration of the insoluble components, as a whole, remained constant. Thus a higher rate of deposition of sequentially extractable component(s) could explain the gradual increase in media's thickness observed in aging human aorta, although the general meaning of this process has not been clearly understood. Conversely, the gradual deposition of insoluble structural components, like collagen fibres, could be partly interpreted as an adjustement of the vessel wall to the decrease in thickness, which would occur in accordance with its radial dilatation.

However, if the total amount of the insoluble components increased proportionally with the progressive radial dilatation of the vessel, their distribution within the aortic media varied considerably with age. In this respect, most prominent was the gradual accumulation, both in relative and absolute terms, of an insoluble protein component, the relative concentration of which almost matched that of elastin in the oldest sample examined.

It has been previously reported that decalcification of mineralized atherosclerotic aorta was accompanied by the release of protein and

and carbohydrate moieties, with the amount of protein released directly related to the initial extent of mineralization (118). Moreover, Keeley (128) studied the non-dialysable components of these extracts and isolated an anionic phosphoglycoprotein, which had the ability of binding cholesterol.

In the present study the relative amount of the total mineral and organic fractions released during decalcification was directly related with age, while that of the undialysable proteins and carbohydrates was not. The age-related increase of the total amount of EDTA/TRIS soluble material was accounted for by low molecular weight diffusible fractions, including components of the mineral phase, calcium and phosphate (98) and small proteins. With respect to the amino acid composition of the phosphoglycoprotein isolated by Keeley (128), the undiffusible fractions showed a lower content in aspartic and glutamic acid, a slightly higher concentration of cysteine, as well as the presence of methionine and hydroxyproline, absent in the former protein. The total hexoses and hexosamines concentrations were quite similar in both preparations. Despite the similarities in the amino acid composition of the undiffusible fractions extracted from different age groups, it was likely that each sample was composed by several different high molecular weight components. Although no attempt was made to assess the distribution of macromolecular species in these preparations, it is well known that high ionic strength solutions like those used for decalcification are able to solubilize several ground substances in addition to cellular components, especially when cell membranes have been previously disrupted by a thorough defatting of the tissue.

With respect to the dry, fat-free decalcified tissue, the total amount of material solubilized with Guan-HCl/TRIS and Guan-HCl/TRIS/DTE was positively correlated with age, while the undiffusible aliquots of both the extracts showed a constant amino acid and carbohydrate composition. However, only the undiffusible fractions extracted with Guan-HCl/TRIS significantly increased with age, as those extracted in the presence of the reducing agent (DTE) did not show any age-related change in concentration. Thus the thickening of human aortic media with age appeared to be partly related to the accumulation of high molecular weight organic fractions which could be solubilized with Guan-HCl/TRIS treatments.

It is noteworthy that similar preparations, obtained by extracting human and animal aorta in the presence of Guan-HCl or urea, have been assumed to represent one homogeneous fraction of the tissue on the basis of their extractability and overall composition, and have been referred to as "structural glycoproteins" (129, 130). However, in the present study, it was evident that the Guan-HCl/TRIS and Guan-HCl/TRIS/DTE extracts contained collagen and proteoglycans in addition to glycoproteins. In particular, about fifteen percent of the undiffusible aliquot of the Guan-HCl/TRIS/DTE extracts could be presumably accounted for by basement membrane collagen (type IV) at least according to their whole amino acid composition (HOPro/HOLys) and solubility properties (131, 132). It appeared that the composition of these extracts was better accounted for by a constant ratio between several different components, rather than by the presence of a prevailing homogeneous fraction of the tissue. It has been previously reported that proteoglycans

(133, 134), smooth muscle proteins and collagen (119) could be extracted using similar procedures from aorta and other connective tissue matrices. It is interesting that the total amount of material solubilized with the sequential extraction from the aortic segments of the youngest age groups corresponded to that which had been extracted from bovine aorta using the same conditions (119).

The insoluble residue of the aortic medial segments was composed of collagen, elastin, and an additional desmosine-free polar protein(s). As previously mentioned, the relative concentration of this residue, as a whole, was age-independent while remarkable age-related changes have been found in the relative proportion of its components.

With age the concentration of collagen rises both in absolute and relative terms. As seen in Table 11, in the first three decades concentrations determined gravimetrically on single samples agree with those calculated from compositional data of pooled specimens. However, in older age groups appreciable differences develop between the two sets of data, the analytical determinations apparently being over-estimated. This anomaly is accompanied by a progressive under-estimation of the concentration of the additional insoluble polar protein component estimated in the same way. Both discrepancies could be explained by the presence of hydroxyproline in the latter matrix constituent. This view is supported by the observation that residues from which collagen was removed by collagenase digestion exhibit an age-related increase in the hydroxyproline to glycine ratio which is matched by an increase in the concentration of polar amino acid residues (Table 8). Contamination by a collagenase-resistant collagen fragment appears unlikely as virtually all known collagens are reduced to small peptides by the action of bacterial collagenase. The only known exception is a high molecular weight fragment of collagen Type IV, known as 7S collagen, which is however readily soluble in the buffers used here (135). It should be pointed out that the distribution of hydroxyproline is not limited to collagen and elastin, this imino acid having been detected in a number of other proteins (136,137 , 138 , 139 , 140). These notably include a non-collagenous protein, similar to osteocalcin (136), which was isolated from the aorta of rats fed on an atherogenic diet (137). Since this protein can potentially bind large amounts of calcium, its identification in human aorta would throw new light on our understanding of the mineralization process of elastic fibres (97 , 98).

The relative concentration of elastin found in the youngest samples was in agreement with that determined by Starcher and Galione (23) in a human aorta of corresponding age. The negative correlation observed between age and elastin's relative concentration has been attributed to the relative increase of the other components of the media rather than to a real decrease of the elastin resulting from degradative processes. Yet the amount of elastin found per unit length of the segments was constant in the whole range investigated. Moreover, the determination of elastin concentration did not take into account that arteries in the body were naturally under condition of longitudinal tension and shorten after removal from the body (14). Hesse found (141) that, in the aorta, there was a retraction of forty percent at age 12, reducing to twelve percent at age 65 and nil at 67.

As the amount of retraction decreased with increasing age, it was evident that in the youngest samples the absolute concentration of the elastin, as well as that of the other medial components, had been at least partly overestimated. Therefore, if we take Hesse's (141) findings into account, it would appear that with ageing there is an absolute increase in the concentration of elastin within the tunica media of the upper thoracic aortic segment under investigation.

The use of collagenase has proved extremely effective in the purification of elastin from bovine tissues (9 , 37 , 42 , 43). When applied to human aorta it produced a preparation with an amino acid profile close to that published by Starcher and Galione (23) (Table 9).only in the case of the 19 years old sample (Table 8). All other preparations required a further digestion with trypsin, an enzyme that does not degrade fully cross-linked elastin, to produce a comparable composition. In several laboratories treatment with hot alkali is still considered, despite its considerable hydrolytic effect on the protein (38), the best purification procedure presently available. Therefore the averaged amino acid composition of three human aortic samples isolated in this way from normal young tissue, plaque areas and non-plaque areas of old aortae has been included in Table 9. When experimentally obtained values of arginine, threonine and serine, reported in brackets, were corrected for hydrolytic losses (35 , 39), differences in polar and hydroxylated amino acid concentrations for enzymic- and alkali-treated preparations were considerably reduced. Such differences can be taken as valuable indicators of protein comtamination. Since the enzyme purification procedure adopted also resulted in a lowering of the hysteresis values, it can be concluded that it was effective in removing components not actively contributing to the elastic recoil. On the other hand,the significant age-independent difference found in the content of proline, alanine and valine, could be a consequence of either differences in their chemical determination or of hydrolytic damages which may have occurred during thermal and alkali treatment.

The invariance in composition of the elastin samples purified with collagenase and trypsin appeared to be incompatible with any appreciable age-related development of cleavages within the highly crosslinked elastin network. This view was corroborated by the identical mechanical responses of all such samples to the applied stress. Therefore, the morphological changes of the elastic lamellae, viz. straightening, thinning and fragmenting, which develop with ageing should perhaps be interpreted as a progressive dispersion of a virtually quantitatively constant and largely undegraded elastin network throughout the wall of an enlarging vessel. The only biochemical evidence for extensive cleavage of elastin polypeptide chains in human aorta comes from studies on old, heavily calcified tissue affected by complex atherosclerotic lesions (142). Therefore, it is reasonable to suppose that degradation of elastin in the tunica media occurs as a result of plaque formation in the intima and is not simply a consequence of ageing. In this respect it is interesting to note that even the 76 years old enzymically purified specimen yielded a stress-strain curve that was normal up to the point of partial failure.

It is worth noting that the human aortic elastin isolated here yielded a non-linear stress-strain curve, while bovine ligamentum nuchae elastin exhibits a linear response to stress (14). The exponential shape observed could be generated by the summation of linear contributions from randomly distributed elastin network segments as they are gradually preferentially oriented in the direction of the applied stress. This view is in keeping with the behaviour of the elastin lamellae, viewed under the microscope, in the intact aorta. These exhibit circumferential folds and waves that progressively diminish with increasing intraluminal pressure up to the diastolic pressure when they all appear to be straight (79).

It has been previously reported that the progressive polar protein contamination of mineralized alkali-treated human aortic elastin was a consequence of the increased presence in the tissue of apatite crystals, which either had simply occluded or prevented the removal of the contaminant by binding to the insoluble elastin component, through alkali-resistant calcium bridges (36). In the present study, however, an alkali- and trypsin-soluble polar protein component appeared to be progressively associated with elastin and to alter its mechanical properties in a manner independent of the inorganic phase which had been previously removed.

The effect of the additional polar protein component on the mechanical properties of elastin can be readily appreciated by comparing the behaviour under stress of aortic rings before and after trypsin digestion. Prior to this treatment, the breaking stress is double that determined after treatment and the incremental modulus shows a rise which is positively correlated with the concentration of the additional protein component. In addition, the stress-strain curves are biphasic with the diagrams becoming linear at progressively lower strains, indicating that the elastin network is more readily oriented in the direction of the applied stress in older samples. Some of the modifications are akin to those brought about by the addition of plasticisers to rubber where the tensile strength and hysteresis values are increased and the strain at rupture is decreased (121).

Rather than altering some basic structural parameters related to the mechanical properties in single connective tissue matrix components such as elastin, the ageing process appears to be associated with the remodeling of the media and with the redistribution of its structural components. Nevertheless, the mechanical properties of intact vessels did not appear to be related simply to the mechanics of single components or to their relative proportion, as interaction may occur between them and thus alter the overall mechanical response of the tissue. Consequently, the elucidation of the structural relationships and interactions between the major components of the aortic tunica media would be very important for the interpretation of the mechanical implications of age-related changes.

SECTION B

MATERIALS AND METHODS

MATERIALS

The bulbus arteriosus and ventral aorta were isolated from specimens of rainbow trout (Salmo gairdneri) and salmon (Salmo salar) with a body weight of about 300 g. Collagenase (EC 3.4.24.3, from Clostridium histolyticum, Type I) and elastase (pancreatopeptidase E, EC 3.4.21.11, from hog pancreas, Type III, chromatographically purified) were purchased from Sigma Chemical Co.. Sodium dodecyl sulfate (specially pure grade) was obtained from B.D.H. Chemicals Ltd., Poole, England. Bio-Gel (Bio-Rad, Richmond, Calif.) with an agarose content of 10% and Sephadex G-10 (Pharmacia) were used as gel chromatography media. Bio-Rad (Richmond, Calif.) AG1-X2 analytical grade anion exchange resin was used in the removal of sodium dodecyl sulfate. 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide meto-p-toluene sulfonate was a product of Aldrich Chemical Co. Ltd., Gillingham, England. All other reagents were obtained from Sigma Chemical Co. and used without further purification.

METHODS

Isolation of bulbus arteriosus and ventral aorta and preparation of specimens for electron and optical microscopy

Samples of rainbow trout and salmon were killed by a crushing blow to the head. Immediately the bulbus arteriosus and ventral aorta were infused with fixative (4% paraformaldehyde - 5% glutaraldehyde in 0.08 M sodium cacodylate, pH 7.3) and excised. Small segments of the isolated bulbus and aorta were removed as shown diagrammatically in Figure 11, and after an additional fixation period were postfixed with 1% osmium tetroxide in 0.08 M sodium cacodylate (pH 7.3), dehydrated in ethanol, and embedded in Araldite, which was cured for 36 h at 60 °C. Ultrathin transverse sections were cut on a Reichardt OMU2 ultramicrotome and stained with 2% uranyl acetate in 50% ethanol for 25 min, followed by 0.4% lead citrate in 0.1 M NaOH for 5 min. Sections were examined in a Zeiss EM9S electron microscope. Specimens for optical microscopy were fixed in Susa's solution according to Heidenhain for 24 h at room temperature and embedded in paraffin. The sections were stained with orcein, haematoxilin-eosin and Azan-Mallory procedures.

Estimation of collagen and elastin content

Segments of the bulbus arteriosus and ventral aorta of trout, taken as indicated in Figure 11, were dried and subsequently hydrolyzed prior to amino and imino acid determination carried out as specified under "chemical analyses". The elastin content was calculated from the concentration of polyfunctional amino acids in the hydrolysate, using the pertinent compositional data presented in Table 12. The collagen content was estimated from the imino acid concentration remaining after deduction of the elastin contribution, using the compositional data published by Mathews (143) for the collagen of carp, which is a closely related teleost.

Purification of elastin

The bulbus arteriosus of specimens of trout and salmon was dissected free from adhering tissue, mechanically comminuted, and washed with 1% (w/w) NaCl at 4 °C for 24 h. Insoluble material was dehydrated and defatted by treatment with ethanol, acetone, and diethyl ether and homogenized prior to extraction for 48 h at 4 °C and 192 h at 18 °C with 5 M Guan-HCl/0.4% EDTA/0.1 M TRIS (pH 7.4), a treatment which removes muscle proteins, collagen, and proteoglycans. This was followed by extraction for 72 h in 5 M Guan-HCl/0.4% EDTA/0.05 M DTE/0.1 M TRIS (pH 7.4) at 37 °C under nitrogen, conditions which ensure solubilization of the microfibrillar component associated with elastin in mammalian tissues (9). Throughout the isolation procedure extraction residues were collected by centrifugation at 24 h intervals and resuspended in fresh extraction medium. The final residues were exhaustively washed with sodium chloride, water, and dried as described in Section A.

Elastin was isolated from bovine ligamentum nuchae as previously described (42).

Preparation of samples of purified elastin for electron microscopy

Pellets of purified elastin were examined in conventionally stained sections following fixation, embedding, and staining as described for fresh tissue. Aliquots of the same preparations were also finely powdered in a vibrating agate ballmill (Vibromill, RIIC) for 60 1-min periods and swollen for 10 min in dimethyl sulfoxyde-containing 1% uranyl formate (14). Ten volumes of 12 mM uranyl formate (adjusted to pH 2.0 with formic acid) were then added, and after 1 h elastin was collected by centrifugation, washed and resuspended in 12 mM uranyl formate (pH 2.0), and finally spread onto carbon coated grids. Grids were examined in a AEI-EM6B electron microscope. Micrographs were taken at instrumental magnifications of 40,000 and 100,000, calibrated using beef liver catalase crystals (144). Suitable micrographs were subjected to optical diffraction analysis.

Digestion of elastin with elastase

Aliquots (50 mg) of purified elastin from trout and salmon bulbus arteriosus and bovine ligamentum nuchae elastin were suspended in 20 ml water, and the pH was adjusted to 8.45 with 0.02 M NaOH. Elastase (500 ug) was added, and proteolysis was carried out under nitrogen at 37 °C in a Radiometer pH stat set at pH 8.45.

Effect of collagenase on salmonid elastin fibrils

Collagenase was purified by treatment with DEAE-Cellulose followed by affinity chromatography on bovine ligamentum nuchae elastin as previously described (42). An aliquot (50 mg) of purified trout elastin was suspended in 20 ml of 0.01 M $CaCl_2$, and the pH adjusted to 7.5 with NaOH. Purified collagenase (500 ug) was added, and the suspension was stirred at 37 °C under nitrogen in a Radiometer pH stat set at pH 7.50. When no further activity was apparent any residue was collected by centrifugation, washed, and lyophilized.

Proteolysis of elastin and peptide fractionation

Aliquots (50 mg) of purified trout, salmon, and bovine preparations were suspended in 16 ml of 0.15% SDS (145). After equilibration for 18 h at 4 °C, the temperature of the suspension was raised to 37 °C, and the pH was adjusted to 8.45 with 0.02 M NaOH. Elastase was added at an enzyme/substrate ratio of 1/500, and the digestion was allowed to proceed at pH 8.45 and 37 °C in a Radiometer pH stat. When there was no further indication of proteolytic activity, the digest was lyophilized. The trout digest was freed of SDS by dissolving it in 5 ml of 8 M urea/0.05 M TRIS/acetate (pH 7.8) to which was added 60 mg of AG1-X2 anion exchange resin (200-400 mesh, Cl^- form) previously washed and equilibrated with the same solvent (146). After stirring for 3 h at 20 °C, the resin was removed by filtration through a 0.45 um MF Millipore filter, and the filtrate was desalted on a column (2.5 x 100 cm) of Sephadex G-10 equilibrated and eluted with water at 4 °C. Effluent preceding the urea front, as determined by refractometry, and exhibiting an optical density at 280 nm in excess of 0.02 was collected and lyophilized. The desalted, detergent-free digest was then dissolved in 2 ml of 0.02 M sodium phosphate buffer (pH 7.0) and applied to a column (2.6 x 70 cm) of agarose (Bio-Gel A-0.5m, 100-200 mesh) equilibrated and eluted at 4 °C with phosphate buffer at a flow rate of 20 ml/h. Effluent was monitored at 280 nm and collected as 10-ml fractions, several of which were hydrolyzed for amino acid analysis.

Chemical analyses

a) Amino acid analysis

Hydrolyses for amino acid analysis were carried out in constant-boiling HCl (1 ml/1 mg protein) containing 0.01 M thioglycolic acid (147) at 110 °C under nitrogen for 24, 36, and 72 h. Analyses were performed on both a JEOL-6AH and a LOCARTE amino acid analyzer. Imino acid concentrations were also determined by a colorimetric procedure (148). Lysine-derived amino acids were identified on both analyzers by the procedures of Volpin and Michelotto (149) and Volpin et al. (150). Dehydromerodesmosine and dehydrolysinonorleucine were estimated as their acid-stable derivative, following reduction of elastin with sodium borohydride (58) prior to acid hydrolysis. The aldol condensation product and α-amino adipic acid δ-semialdehyde were determined as their alcohol derivatives in alkaline hydrolyzates of reduced elastin (21). ε-hydroxynorleucine was found to emerge immediately in front of glycine on the LOCARTE analyzer without modification of the buffer system. Its elution volume and colour value were determined using a pure sample of the compound, synthesized according to Gaudry (151).

b) Determination of amide content

Aliquots (12 mg) of purified elastin fibrils from salmon bulbus arteriosus and bovine ligamentum nuchae were suspended in 4 ml of 7.5 M urea/ 1.33 M norleucine methyl ester-HCl, and the pH was adjusted to 4.75. Following an 18 h equilibration period at 18 °C, the temperature of the suspension was raised to 25 °C, and a 0.4 M solution of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate in 7.5 M urea was added to give a reagent concentration of 0.1 M. The reaction mixtures were stirred for 18 h at 25 °C, the pH being maintained at 4.75 by the addition of 0.1 M HCl. Elastin fibrils were collected by centrifugation and exhaustively washed with 1 mM HCl followed by 7.5 M urea and water, prior to acid hydrolysis for amino acid analysis. Amide content was calculated from the concentration of free β- and γ-carboxyl groups, as estimated by their coupling with norleucine, and the total content of aspartic and glutamic acid residues (152).

Absorption and fluorescence spectroscopy

The UV absorption spectra were determined on elastase digests of both trout and ligamentum nuchae preparations and fluorescence spectra only on those of trout, dissolved in 0.1 N NaOH or 0.1 N HCl at a concentration of either 0.5 or 1.0 mg/ml. The UV spectra were recorded with an HITACHI-PERKIN ELMER spectrophotometer, Model 124, while fluorescence was measured with a PERKIN ELMER spectrofluorimeter, MPF-4, coupled with a PERKIN ELMER recorder, Model J6. Samples were excited at 253.5 nm using slit 811 and an instrumental sensitivity of 1. Spectra were corrected for solvent fluorescence.

Dynamometry

The intact bulbus arteriosus of salmon was subjected to the purification procedure described in "purification of elastin" section above. A thick (about 5 mm) section, approximating a cylinder of uniform diameter, was removed from each bulbus , and its dry weight and dimensions were recorded. The two cut surfaces were then coated with epoxy resin, and the specimen was mounted between two metal disks, each provided with a central hole to allow the free passage of liquid through the section. After equilibration in water for 48 h, the dimension of each specimen were again recorded before it was connected to the testing instrument described in section A by means of fine metal chain attached to the metal disks. The specimen was immersed in water at 20 °C and extended at a rate of 0.1 mm/sec, the outputs of the helipot and the transducer being fed into an XY-recorder.

As the relationship between stress and strain was linear over the whole range investigated, the Young's modulus (E_Y) was calculated directly on the obtained stress-strain diagram according to the equation

$$E_Y = \frac{F / A}{\Delta L / L_o}$$

where F/A is the tensile stress, expressed as force per unit area, while $\Delta L/L_o$ is the tensile strain. (A) correspond to the cross-sectional area and L_o to the length of the unstretched mounted sample, respectively, and F is the force applied to the specimen.

The cross-sectional area of each hydrated sample was expressed as the ratio between the volume of the dry sample and the length determined after equilibration in water. The volume of the dry sample was estimated according to its dry weight and the density calculated from the amino acid composition (120).

The mean inter-crosslink chain molecular weight (Mc) was evaluated from tensile strength measurements by adopting the following formula (153)

$$f = (RT\varrho \nu_2^{1/3} / Mc)\ (\alpha - [1/\alpha^2])$$

where f is the retractive force per unit area; α is the extension of the material, i.e., the stretched length divided by the initial length; ϱ is the density of the dry elastomer; v_2 is the volume fraction of the elastomer in the composition which is being stretched; and R is the gas constant.

Wide-angle X-ray analysis

Purified elastin fibrils from salmon and trout bulbus arteriosus were examined in the dry state in a PYE-UNICAM cylindrical camera (diameter of collimators, 1.14, 0.53 and 1.02 mm; sample to film distance, 30 mm). Scattering patterns were obtained with nickel-filtered CuKα radiation using an exposure time of 100 min.

RESULTS

Optical microscopy

In paraffin sections, the structure of both the bulbus arteriosus and the ventral aorta was very similar in trout and salmon. The wall of the vessel comprised an endothelium and several layers of smooth muscle cells (Figure 11 and 13) embedded in a matrix which was characterized by the presence of fibres which showed a strong affinity for orcein stain and had a diameter of approximately 2-4 μm (Figure 12 and 14). The ventral aorta exhibited two approximately equal layers of smooth muscle cells, those of the inner layer being oriented circumferentially while the other was directed longitudinally, with respect to the main vessel axis. In the bulbus arteriosus an additional layer of longitudinally oriented smooth muscle cells was found under the endothelium. In general, the orcein stained fibres appeared to be arranged in a continuous threedimensional array the major fibrous bundles running approximately parallel to the main axis of the smooth muscle cells.

Electron microscopy

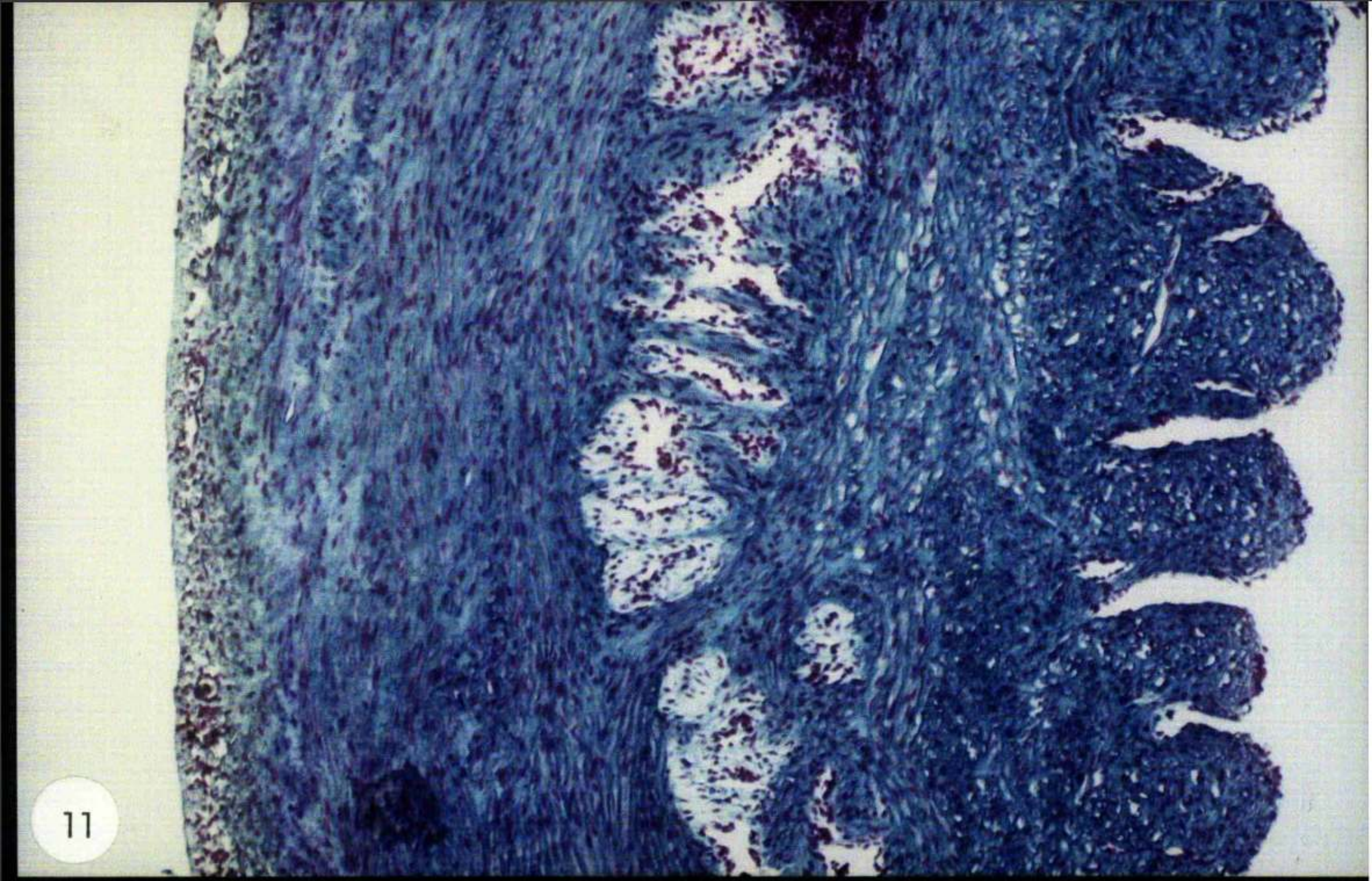
The matrix between the smooth muscle cells was characterized by the presence of fibrils which showed strong affinity for electron-dense cations and had a diameter of about 25 nm (Figure 15, 16 and 17). The muscle cells showed extensive intercellular contacts, Figure 18, characterized by junctions (arrowhead in Figure 18) which have been tentatively classified as being of the nexus type. The overall thickness of the membrane complex is, in fact, in these areas twice that of the neighbouring plasma membrane (154). The majority of the 25 nm fibrils exhibit no preferential orientation and are interwoven at random to form what appears to be an isotropic

F I G U R E 11

Transverse section of trout bulbus arteriosus, stained according to the Azan-Mallory procedure. X 105. Optical microscopy.

F I G U R E 12

Transverse section of trout bulbus arteriosus. Orcein stained elastic fibres. X 105. Optical microscopy.



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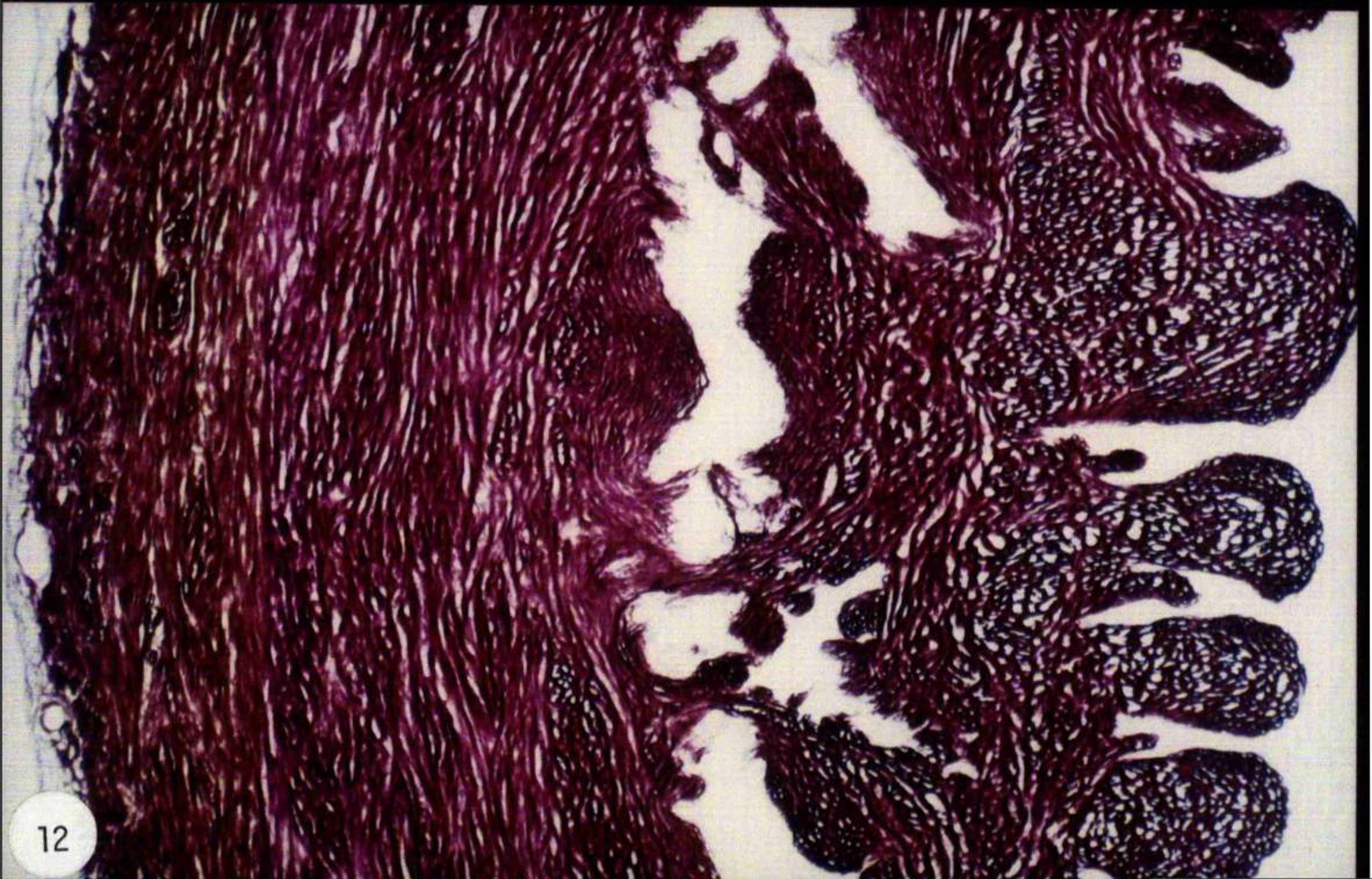


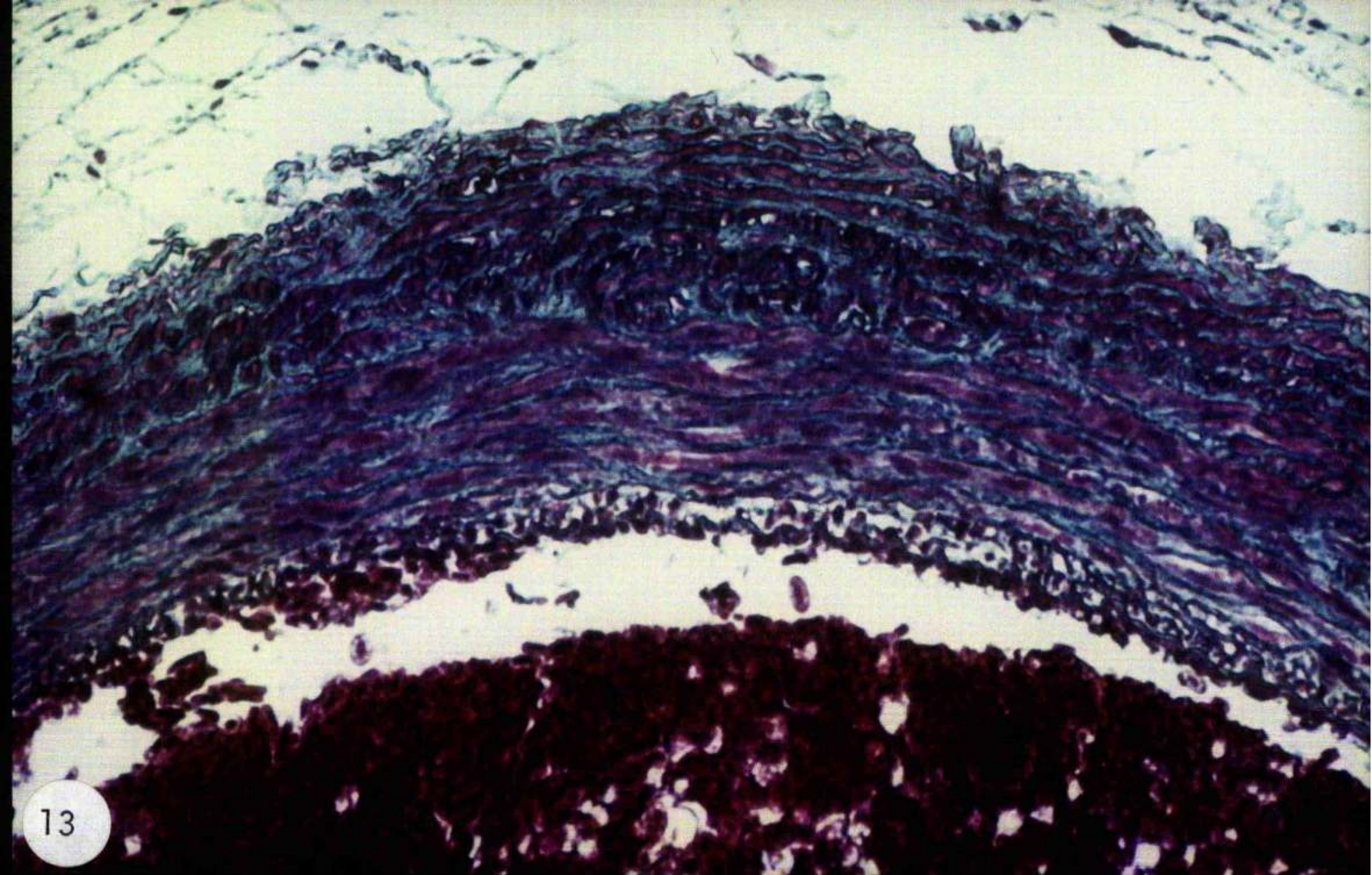
12

FIGURE 13

Transverse section of trout proximal ventral aorta, stained according to the Azan-Mallory procedure. X 266. Optical microscopy.

FIGURE 14

Transverse section of trout proximal ventral aorta. Orcein stained elastic fibres. X 266. Optical microscopy.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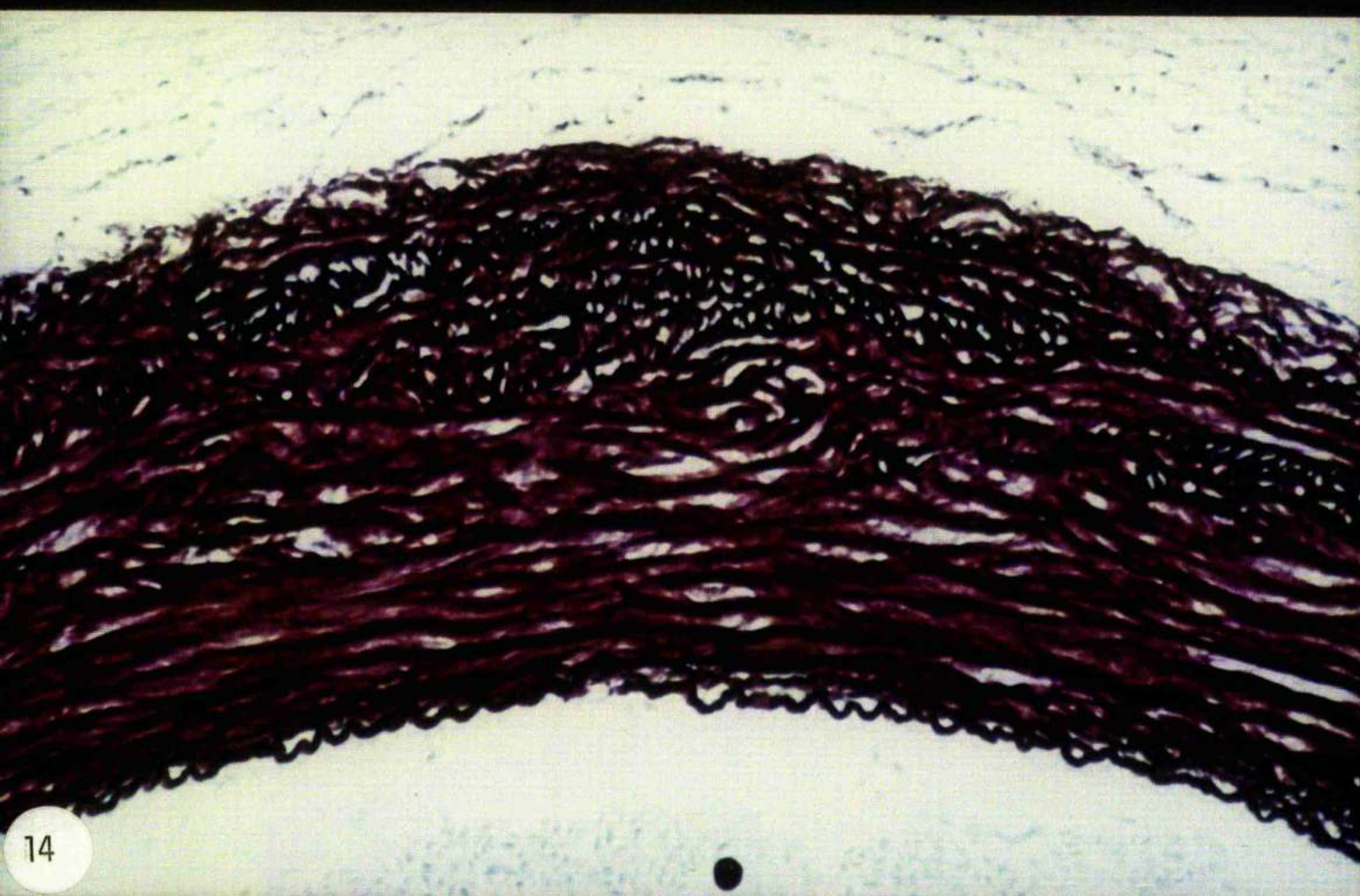
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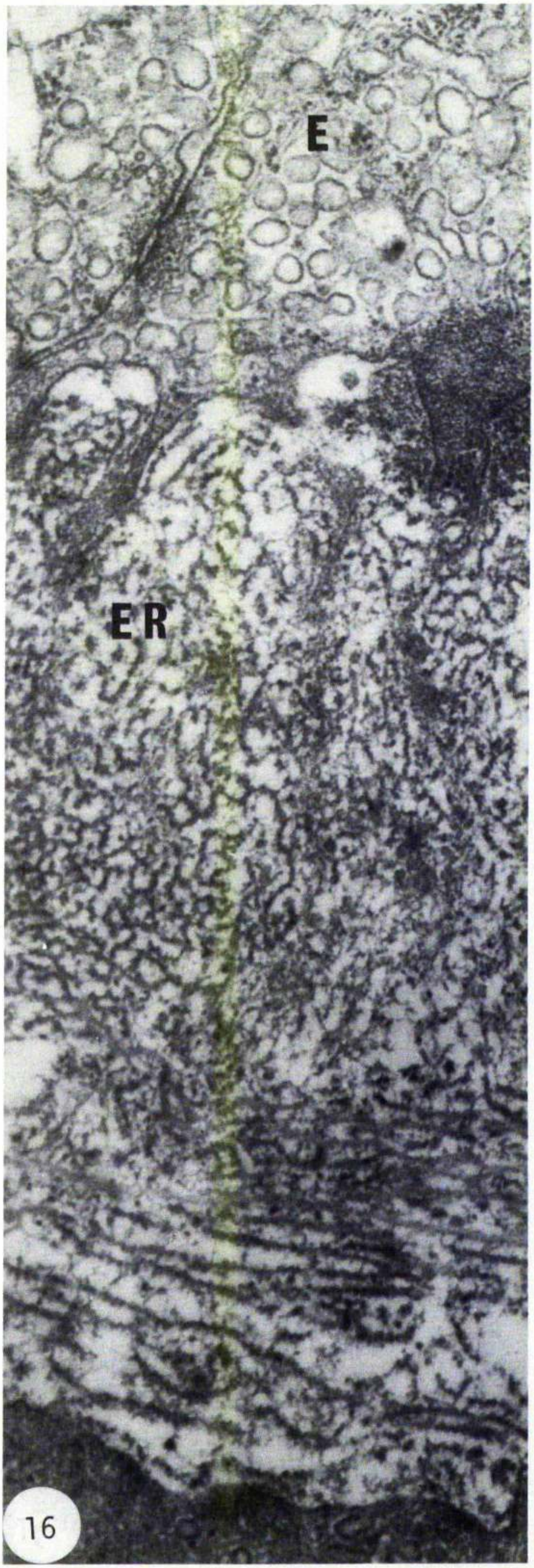
FIGURE 15

Transverse section of salmon bulbus arteriosus, stained with uranyl acetate-lead citrate. X 7700. Electron microscopy.

FIGURE 16

Transverse section of trout ventral aorta, close to the distal bifurcation, stained with uranyl acetate-lead citrate.
E, endothelial cell; ER, subendothelial elastin reticulum. X 44 500.
Electron microscopy.


15

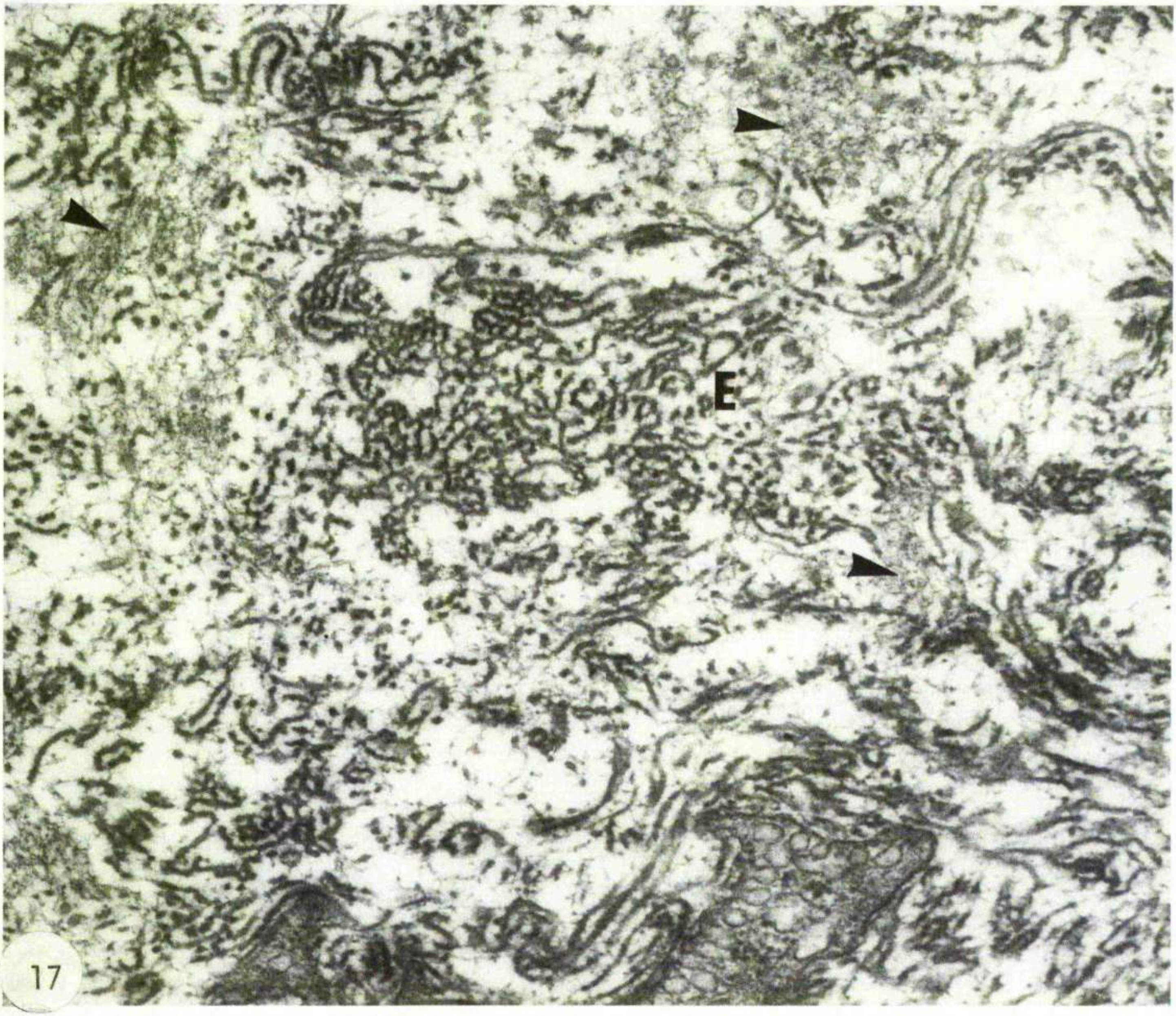

E
ER
16

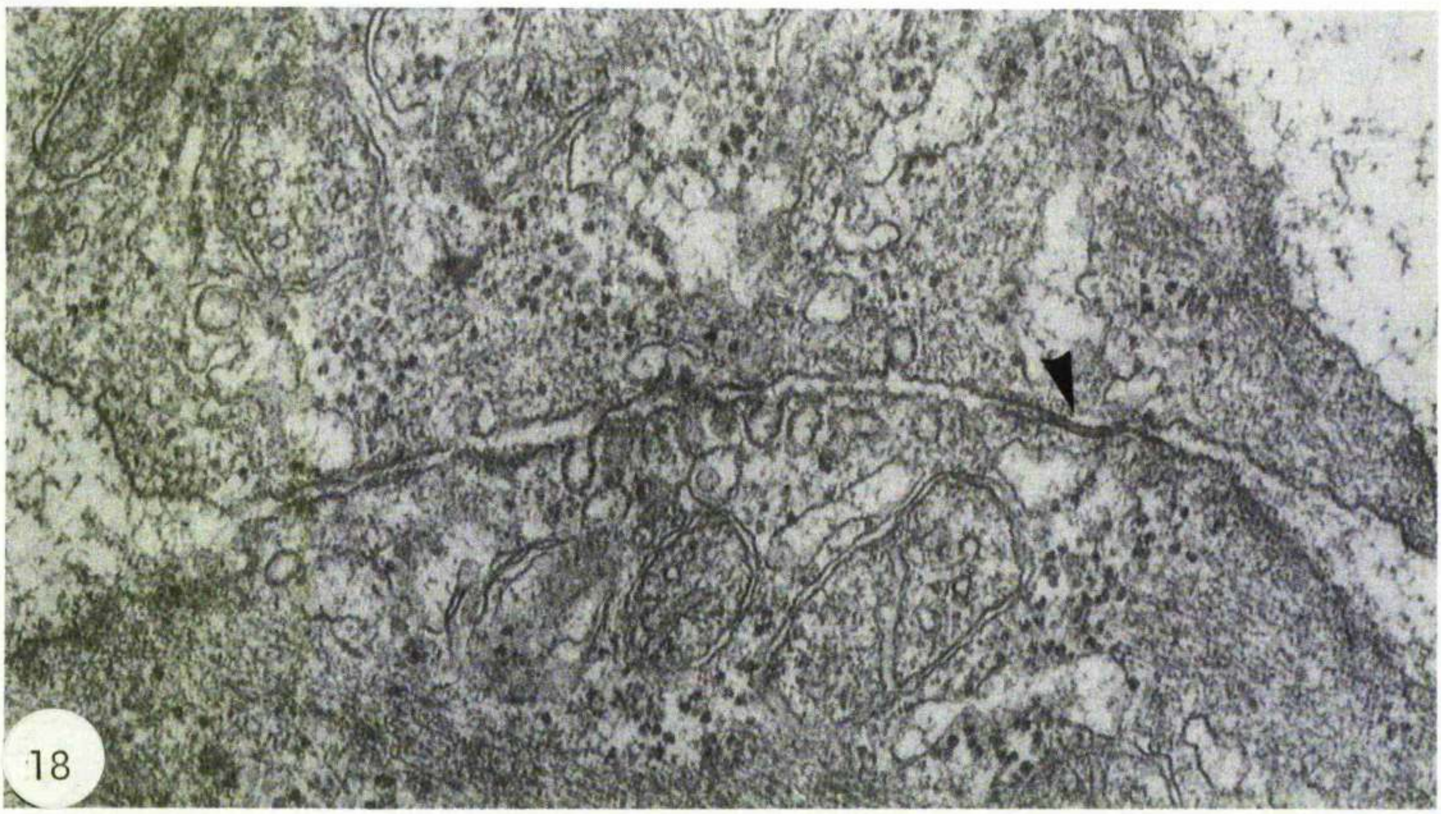
F I G U R E 17

Transverse section of salmon bulbus arteriosus, stained with uranyl acetate-lead citrate. Intercellular matrix containing elastic fibrils (E) and a fine, electron-dense reticular structure (arrowsheads). X 36 500. Electron microscopy.

F I G U R E 18

Transverse section of trout bulbus arteriosus, stained with uranyl acetate-lead citrate. Intercellular contact between two muscle cells. Arrowhead indicates gap junction. X 50 800. Electron microscopy.


E
17


18

threedimensional system (Figure 17). This pattern is present throughout the vessel wall, although the fibrils located between the endothelium and the first layer of muscle cells,(Figure 16) are more densely packed than those in a more peripheral location. Immediately beneath this compact subendothelial layer (Figure 16), and occasionally elsewhere in the media and adventitia (Figure 19 and 21), the fibrils exhibit some degree of orientation and lateral alignment. Often in such areas the fibrils appear to coalesce and to lose some of their affinity for stain. Where such an arrangement is more pronounced (Figure 20), the appearence of the resultant bundle of fibrils is almost amorphous and remarkably similar to that of elastic fibres in mammalian tissues.

The collagenous component of the bulbus arteriosus and ventral aorta is mainly confined to the adventitia, although it is occasionally seen in the media and, infrequently, under the compact subendothelial layer. In all such locations it is present in the form of bundles of 40 nm diameter fibrils which are predominantely aligned with the long axis of the vessel (Figure 21).

In addition to the two fibrous components already mentioned, the intercellular spaces are occupied by an extremely fine reticulum which probably represents proteoglycans and glycoproteins precipitated during fixation.

Of these morphologically identifiable matrix constituents, the 25 nm fibrils alone are present in the residue remaining after extraction with Guan-HCl/TRIS and Guan-HCl/TRIS/DTE (Figure 22, 23 and 24). Although these fibrils appear to be identical to those present in the intact tissue, the

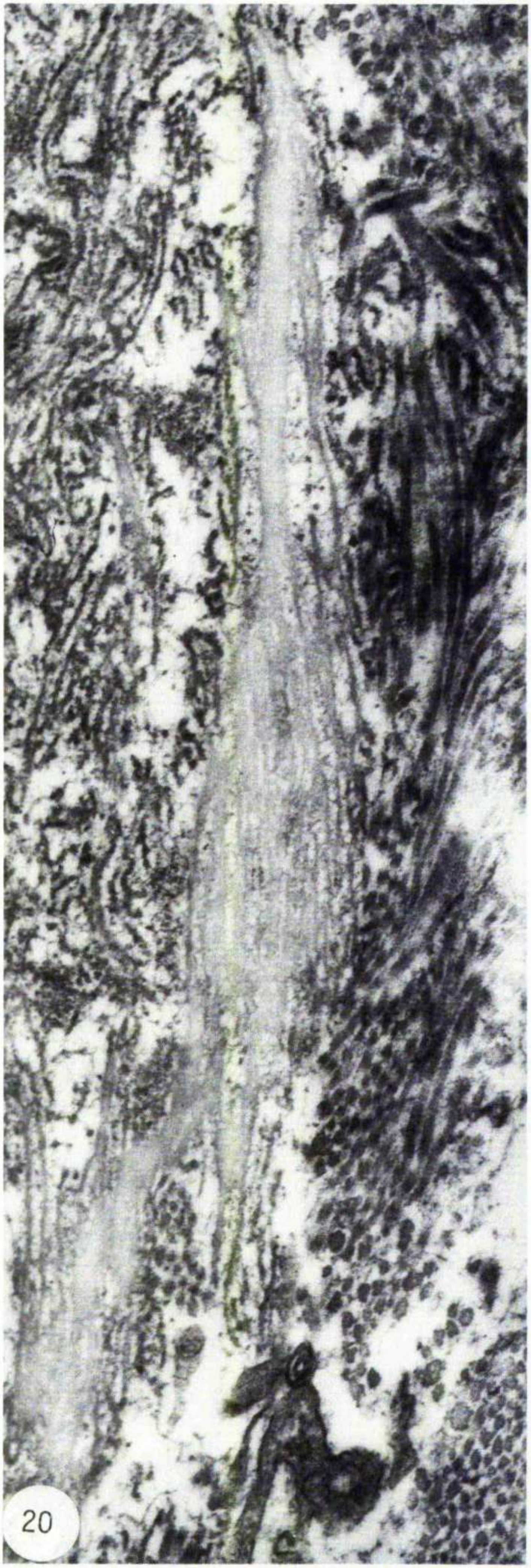
FIGURE 19

Transverse section of salmon bulbus arteriosus, stained with uranyl acetate-lead citrate. Intercellular matrix containing aligned elastic fibrils. X 31 200. Electron microscopy.

FIGURE 20

Transverse section of salmon ventral aorta, close to the distal bifurcation, stained with uranyl acetate-lead citrate. Intercellular matrix containing aligned and partially fused elastic fibrils. Collagen fibrils are visible on the right-hand side of the plate. X 43 000. Electron microscopy.


19


20

F I G U R E   21

Transverse section of salmon ventral aorta, close to the first branch point, stained with uranyl acetate-lead citrate. Intercellular matrix containing bundles of collagen fibrils running perpendicular to the plane of section. X 37 300. Electron microscopy.

F I G U R E   22

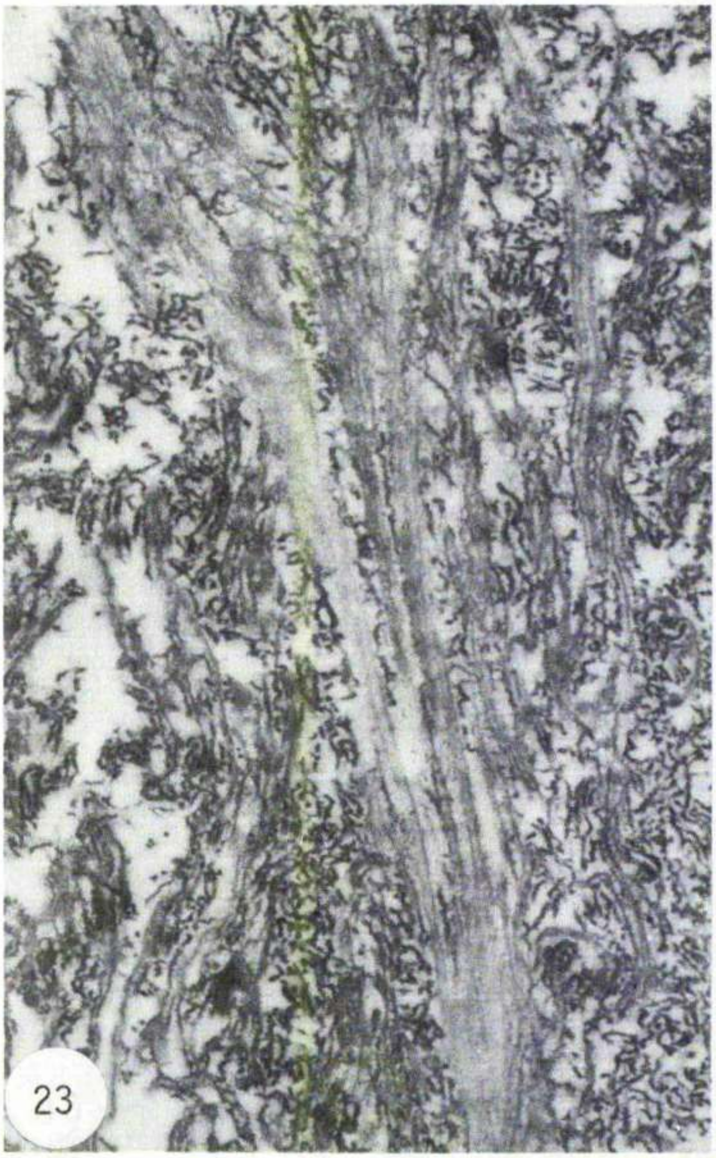
Section of embedded, purified elastin fibrils from salmon, stained with uranyl acetate-lead citrate. X 44 500. Electron microscopy.

F I G U R E   23

Section of embedded, purified elastin fibrils from salmon, stained with uranyl acetate-lead citrate. Note the presence of aligned fused fibrils. X 22 800. Electron microscopy.

21

22



23

F I G U R E 24

Section of embedded, purified elastin fibrils from trout, stained with uranyl acetate-lead citrate. X 67 500. Electron microscopy.

F I G U R E 25

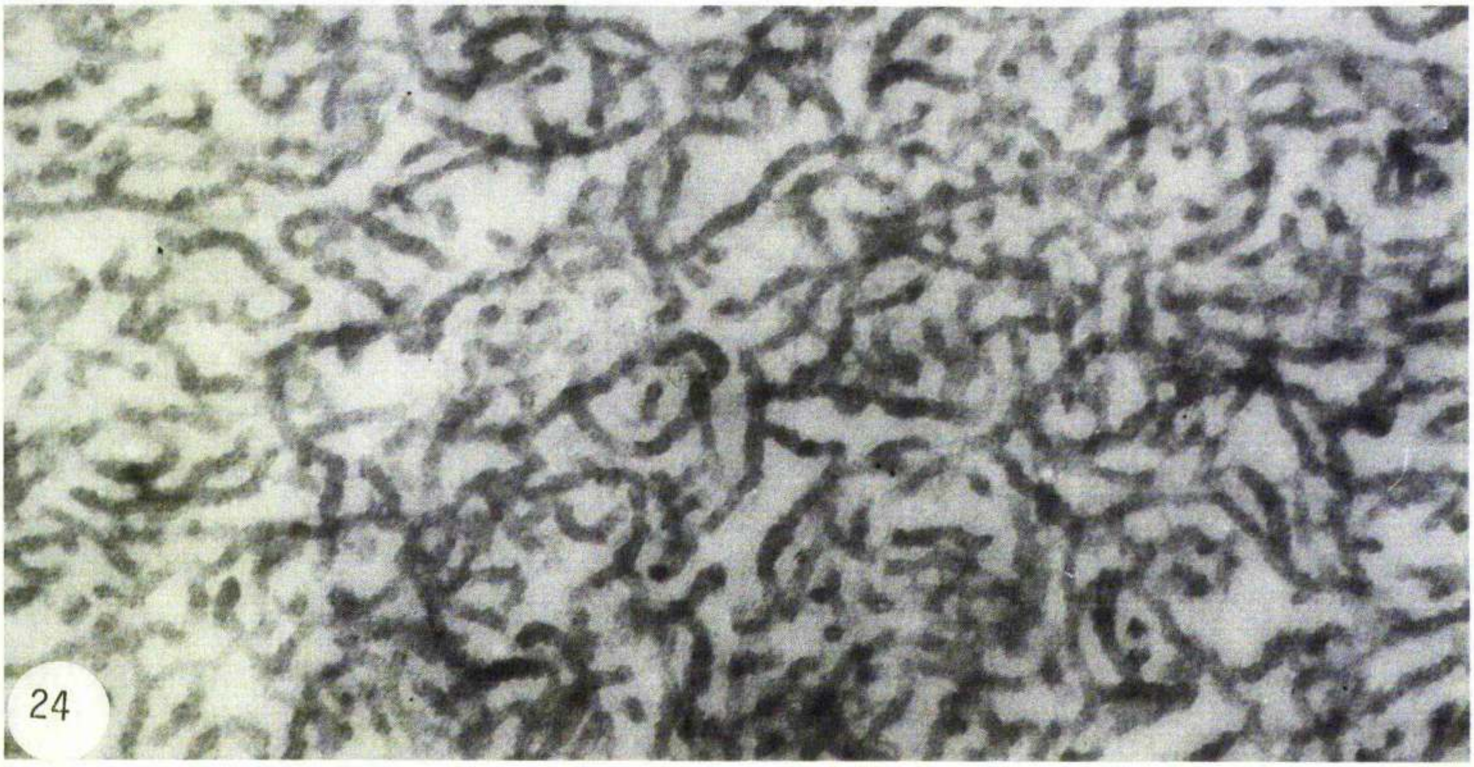
Elastin fibril from trout, negatively stained with uranyl formate (pH 2.0). X 780 000. Electron microscopy.

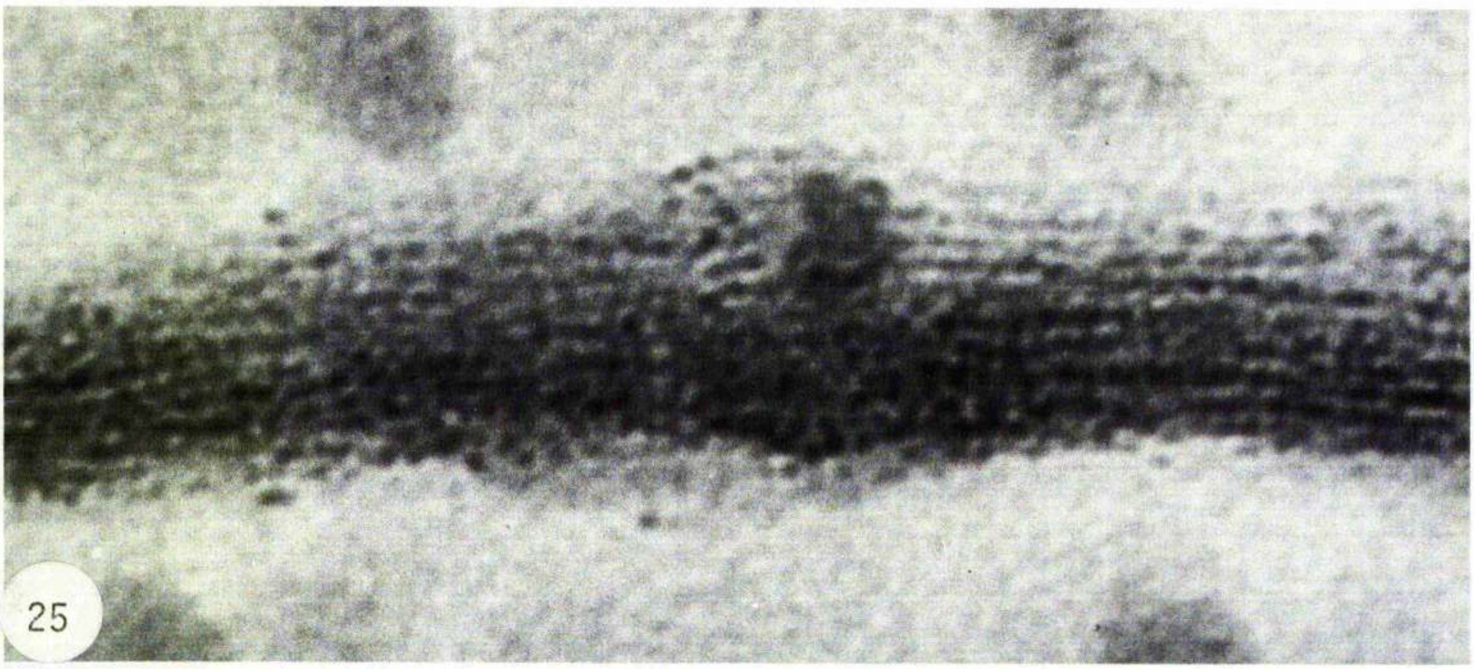
F I G U R E 26

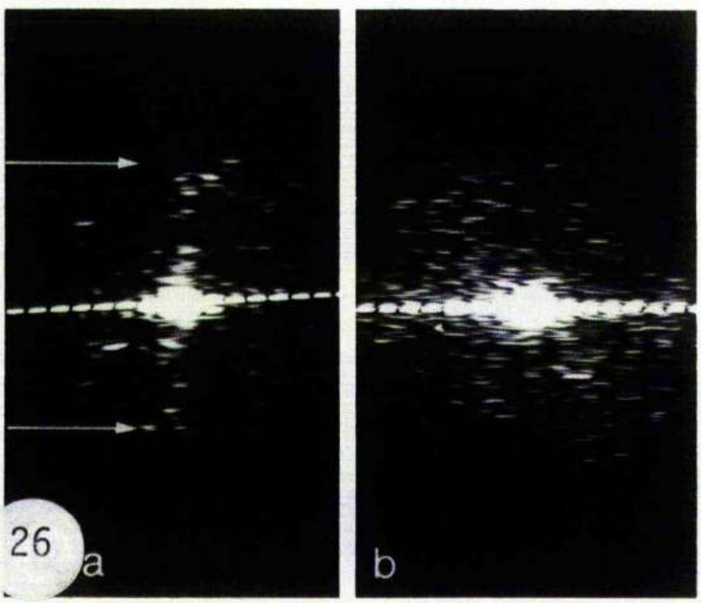
Optical diffraction patterns of negatively contrasted trout elastin, generated by the fibril shown in Figure 25. (a) Equatorial and (b) meridional patterns. The pair of reflections indicated by arrows corresponds to a spacing of 5.5 nm.

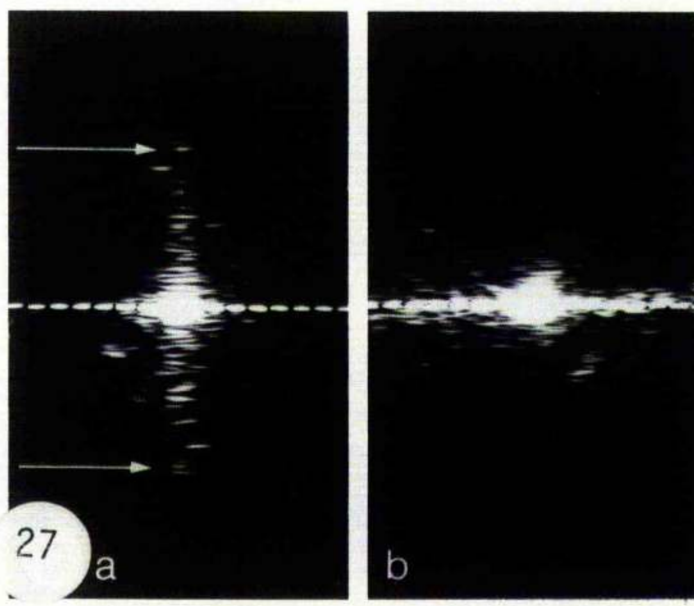
F I G U R E 27

Optical diffraction patterns generated by a negatively contrasted fibril of purified elastin from bovine ligamentum nuchae. (a) Equatorial and (b) meridional patterns. The pair of reflections indicated by arrows corresponds to spacing of 4.5 nm.


24


25


26
a
b


27
a
b

frequency with which they coalesce is much greater than that assessed prior to their isolation.

Figure 25 shows the typical appearence of a negatively contrasted fibril resolved into its constituents primary filaments, the diameter of which can be estimated to be about 2.5 nm. The diameter of fibrils in such preparations was found to average 35 nm. The discrepancy between this value and that determined in embedded preparations is probably attributed to a cross-sectional distortion owing to the spreading effect of surface tension forces operative during the drying-down phase in the preparation of negatively contrasted specimens. The equatorial (a) and meridional (b) optical diffraction patterns produced by one such fibril are shown in Figure 26, while those generated by an equivalent fibril from bovine ligamentum nuchae are shown in Figure 27. In both equatorial patterns arrows indicate diffractions corresponding to the shortest periodicities detectable. These were 5.5 nm for the salmonid specimen and 4.5 nm for the bovine preparation.

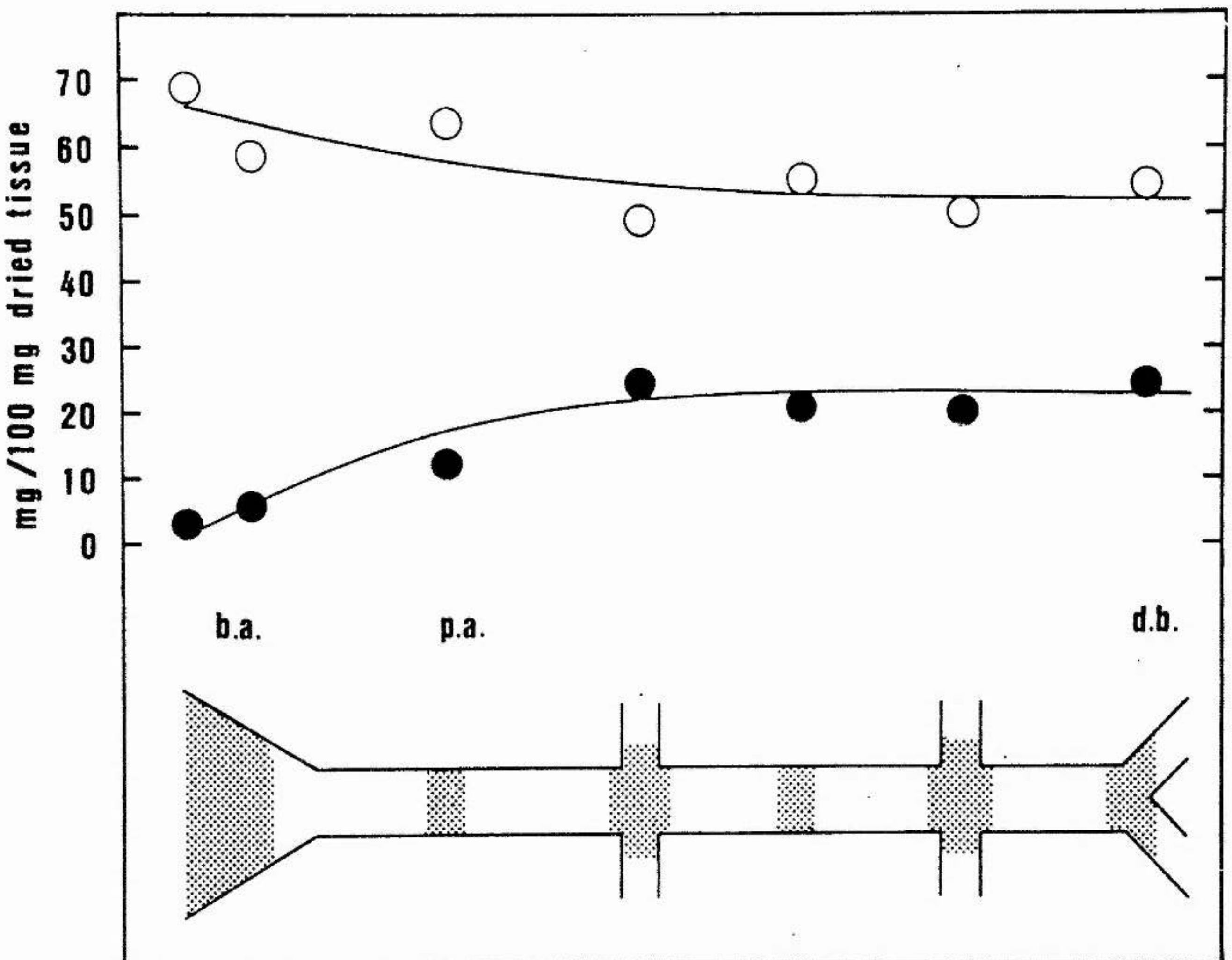
Distribution of elastin and collagen in trout bulbus arteriosus and ventral aorta

Data presented in Figure 28 show that in trout there was a gradient of diminishing elastin content from the bulbus arteriosus to the ventral aorta. A corresponding reverse gradient operated for collagen which was almost absent in the major part of the bulbus where the concentration of elastin corresponded to about 70% (w/w). At the level of the distal bifurcation of the ventral aorta elastin concentration decreased to 55% (w/w) while that of collagen rose to a value of 25% (w/w).

F I G U R E 28

Distribution of elastin (O) and collagen (●) in the bulbus arteriosus and ventral aorta of trout. Stippled areas in the diagrammatic representation of the blood vessel correspond to the segments used for chemical and electron microscopic analysis. b.a., p.a., and d.b. stand for bulbus arteriosus, proximal aorta, and distal bifurcation, respectively.

FIGURE 28



This pattern of nonuniform distribution of scleroproteins was in conformity with data reported for other species (72, 110, 155) and probably reflected variations in the functional activity of different portions of the vessels.

Amino acid composition of elastic fibrils isolated from trout and salmon bulbus arteriosus

The amino acid composition of trout and salmon purified elastic fibril preparations are reported in Table 12, together with that of bovine ligamentum nuchae for comparison. Salmonid elastins showed a much higher content of hydrophilic amino acids, higher glycine (about 430 residues/1000 amino acid residues) and lower alanine and valine content. It is noteworthy that the hydroxyproline content was lower in salmonid preparations with respect to the bovine protein. Apart from their chemical structure, the total number of crosslinking compounds accounted for 1.5 residues/1000 amino acid residues in trout, 2.1 in salmon and 7.0 in bovine elastin. Particularly the tetrafunctional crosslinking amino acids (desmosine plus isodesmosine) accounted for 0.7, 0.4 and 3.9 residues/1000 amino acid residues,respectively. The molar ratio between the tetrafunctional and the bifunctional crosslinks was 0.84 in salmon, 0.23 in trout, and 1.33 in bovine elastin.

Moreover, in bovine and salmon elastin eighty and ninety percent, respectively, of total aspartic and glutamic acid residues was found in amide form. It is of interest that salmonid elastin fibrils were also enriched in α-amino adipic acid δ-semialdehyde recovered as ε-hydroxynorleucine.

TABLE 12

AMINO ACID COMPOSITION OF ELASTIC FIBRILS FROM TROUT AND SALMON BULBUS ARTERIOSUS AND BOVINE LIGAMENTUM NUCHAE

SOURCE / AMINO ACID	Trout bulbus arteriosus	Trout bulbus arteriosus collagenase digest residue	Salmon bulbus arteriosus	Bovine ligamentum nuchae
Hydroxyproline	6.7	6.4	5.6	8.5
Aspartic acid	23.0	18.5	28.2	5.7
Threonine	31.8	28.8	31.1	9.2
Serine	31.5	31.6	33.3	8.6
Glutamic acid	37.6	40.1	40.0	15.1
Proline	90.9	88.0	86.3	114.0
Glycine	444.7	448.5	420.3	336.2
Alanine	141.5	141.4	156.6	224.0
Valine	46.9	51.3	49.0	129.9
Half-cystine	0.0	0.0	0.0	0.0
Methionine	4.6	4.4	6.0	0.0
Isoleucine	9.3	8.2	10.4	23.6
Leucine	35.2	38.9	35.1	58.6
Tyrosine	43.1	45.1	34.8	5.8
Phenylalanine	11.0	11.5	12.3	28.9
Hydroxylysine	0.0	0.0	0.0	0.0
Lysine	7.8	4.7	10.9	3.2
Histidine	6.3	5.2	8.0	0.4
Arginine	22.8	21.3	25.8	5.7
ε-Hydroxynorleucine	1.1	ND	1.2	0.6
Aldol condensation product*	1.2	ND	2.1	3.7
Dehydrolysinonorleucine*	0.3	ND	0.1	0.0
Lysinonorleucine*	0.1	0.1	0.9	2.1
Dehydromerodesmosine*	0.0	ND	0.6	0.1
Merodesmosine*	0.0	0.0	0.0	0.5
Isodesmosine*	1.7	2.8	0.8	5.4
Desmosine*	1.0	1.8	0.6	10.1
Amide	ND	ND	55.4	16.6

Values are expressed as residues/1000 total amino acid residues.

* Expressed as lysine equivalents

Proteolysis of elastic fibrils

Exposure of elastic fibrils to a purified preparation of collagenase, which produced no significant degradation of bovine elastin (42), led to the solubilization of approximately 80% of the salmonid preparation. The amino acid composition of the undigested material is reported in Table 12. With respect to the original untreated protein, significant variations were observed only among lysine content which halved, and that of desmosine plus isodesmosine which doubled.

Elastase digestion of trout, salmon and bovine elastic fibrils at an enzyme/substrate ratio 1/100, in the absence of SDS, resulted in their complete solubilization. The digestion progression curves for salmonid elastic fibrils and bovine elastin are reported in Figure 29. According to the alkali uptake, in both salmon and trout elastin the total number of peptide bonds cleaved by the action of elastase was about half that cleaved in the same conditions in bovine elastin. Nevertheless the time progression of the degradation process appeared to be very similar in all the samples.

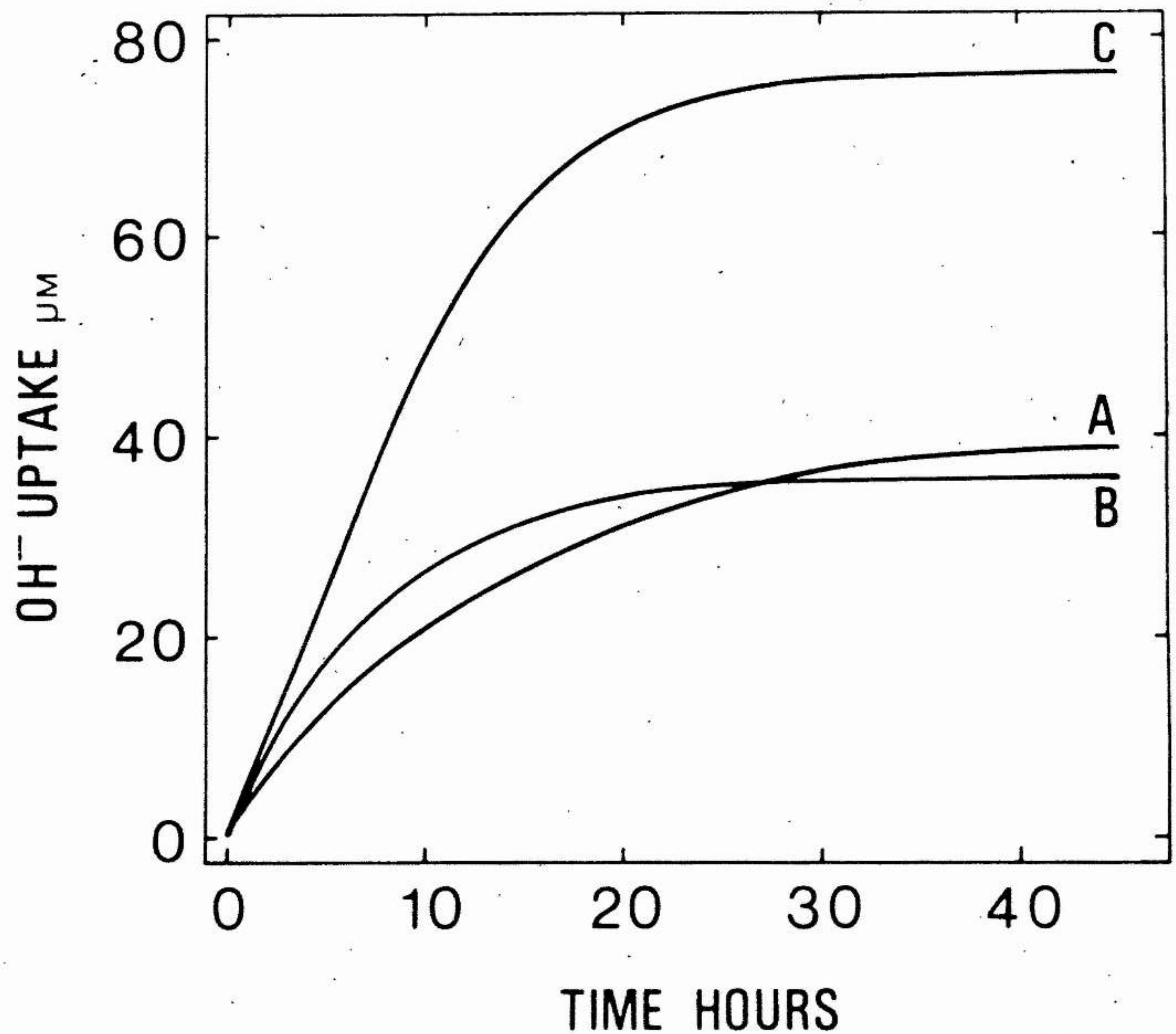
Fractionation of peptides obtained by elastase digestion of trout elastic fibrils in the presence of SDS

In the presence of SDS elastolysis of trout, salmon and bovine preparations resulted in their complete solubilization. The digestion progression curves are reported in Figure 30. Following removal of detergent, the fractionation of the trout digest produced the elution profile shown in Figure 31. The soluble peptides obtained by enzymic degradation of trout elastic fibrils were eluted as two major broad peaks partially overlapped

FIGURE 29

Progress curves of the digestion with elastase of salmon (A) and trout (B) elastic fibrils and bovine elastin (C) in the absence of sodium dodecyl sulfate. Alkali uptakes have been normalized to a 50 mg aliquot of substrate.

FIGURE 29



F I G U R E 30

Progress curves of the digestion with elastase of salmon (A) and trout (B) elastic fibrils and bovine elastin (C) in the presence of sodium dodecyl sulfate. Alkali uptakes have been normalized to a 50 mg aliquot of substrate.

FIGURE 30

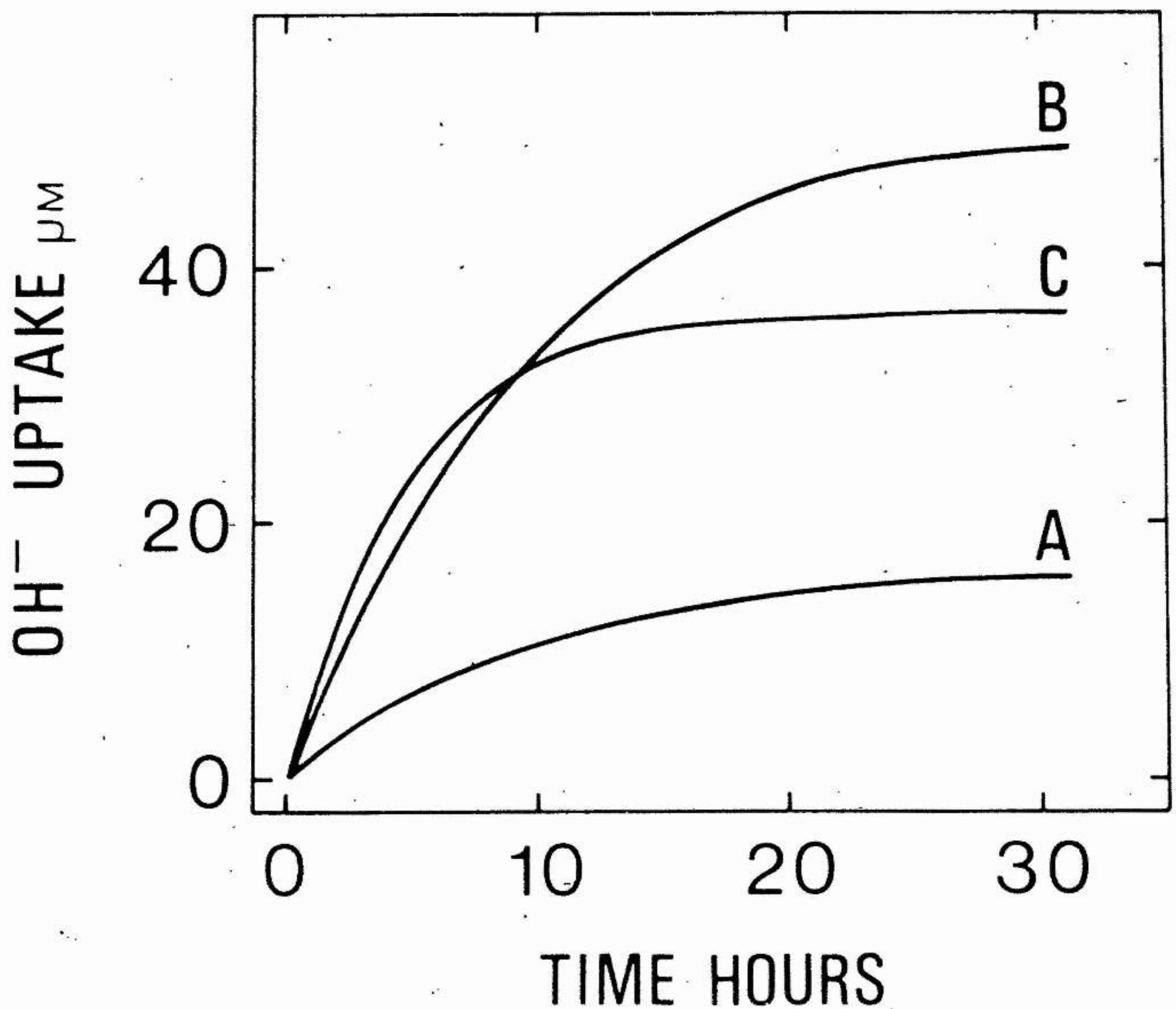
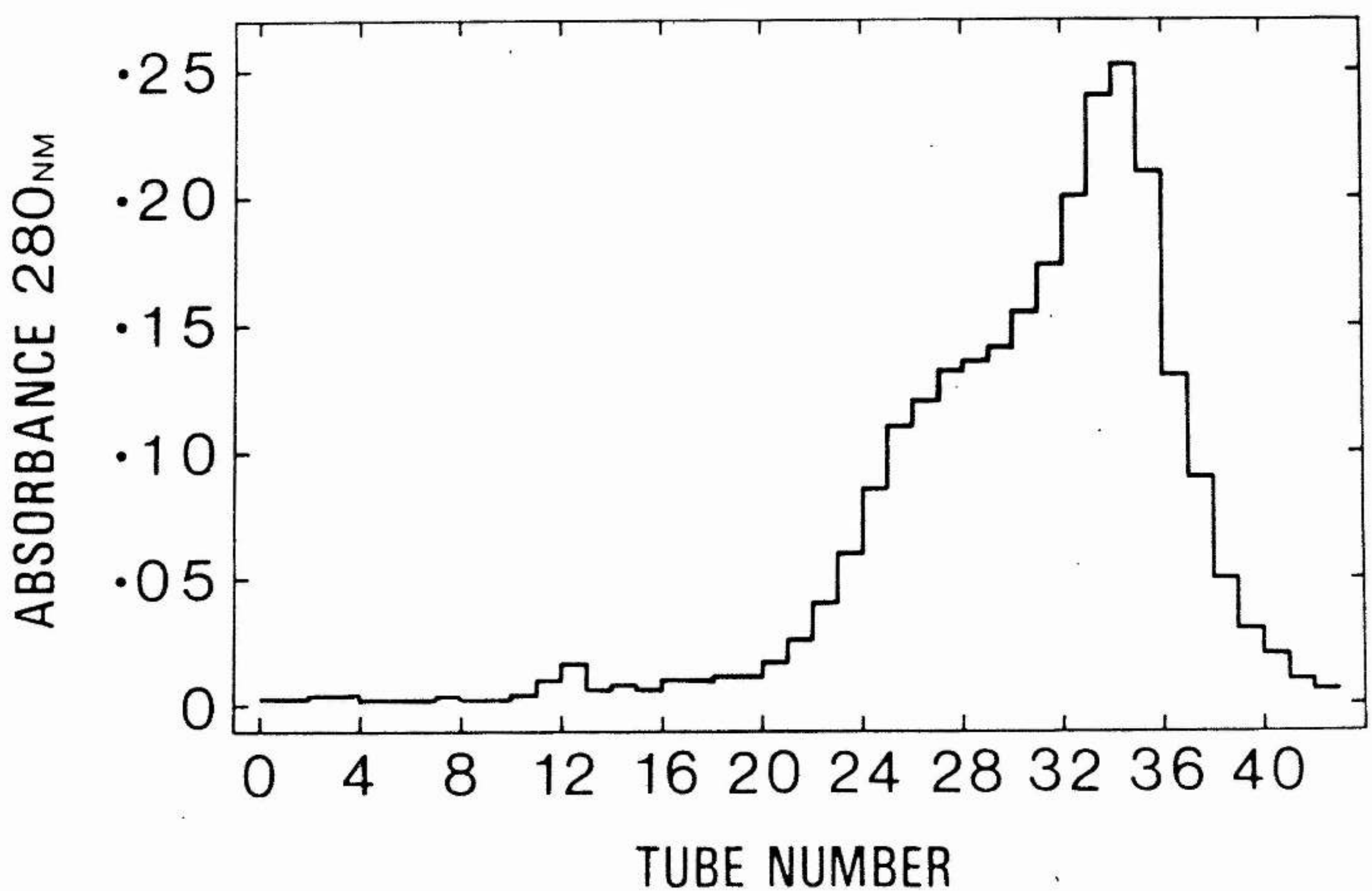


FIGURE 31

Elution profile of peptides obtained by elastase digestion of trout elastic fibrils in the presence of sodium dodecyl sulfate on agarose. Fraction volume, 10 ml.

FIGURE 31


ABSORBANCE 280NM
·25
·20
·15
·10
·05
0
0 4 8 12 16 20 24 28 32 36 40
TUBE NUMBER

and a minor peak which corresponded to the void volume of the column. The protein fractions corresponding to the centre part of each peak, namely tube number 13, 26 and 35, were hydrolyzed and their amino acid composition was determined and reported in Table 13. The overall recovery of starting material after digestion, detergent removal and agarose fractionation was about 50%.

The high molecular weight fraction corresponding to the void volume of the column, tube number 13, represented only 0.5% of the starting material, and showed an amino acid composition totally at variance with the rest of the preparation and was devoid of crosslinking compounds. With respect to the amino acid composition of the untreated protein, the fraction 26 exhibited a fourfold increase of the crosslinks as well as a thirty per cent increase of the alanine content, while in fraction 35 the concentration of hydrophilic amino acids was lower and that of glycine higher.

Absorption and fluorescence spectroscopy

The UV absorption spectra of the elastase digest of trout and mammalian preparations are reported in Figure 32. Of particular relevance in the case of the salmonid digest was the absence of an absorption band, in the sample examined under alkaline conditions in the 317- to 322-nm region, where di-tyrosine, tri-tyrosine, and enzymic digests of resilin exhibited pronounced absorptions (156). The major absorption peak was presumably due to tyrosine as it showed the characteristic 293- to 275-nm pH-dependent shift.

When excited at 253.7 nm, di-tyrosine, and native resilin all exhibited

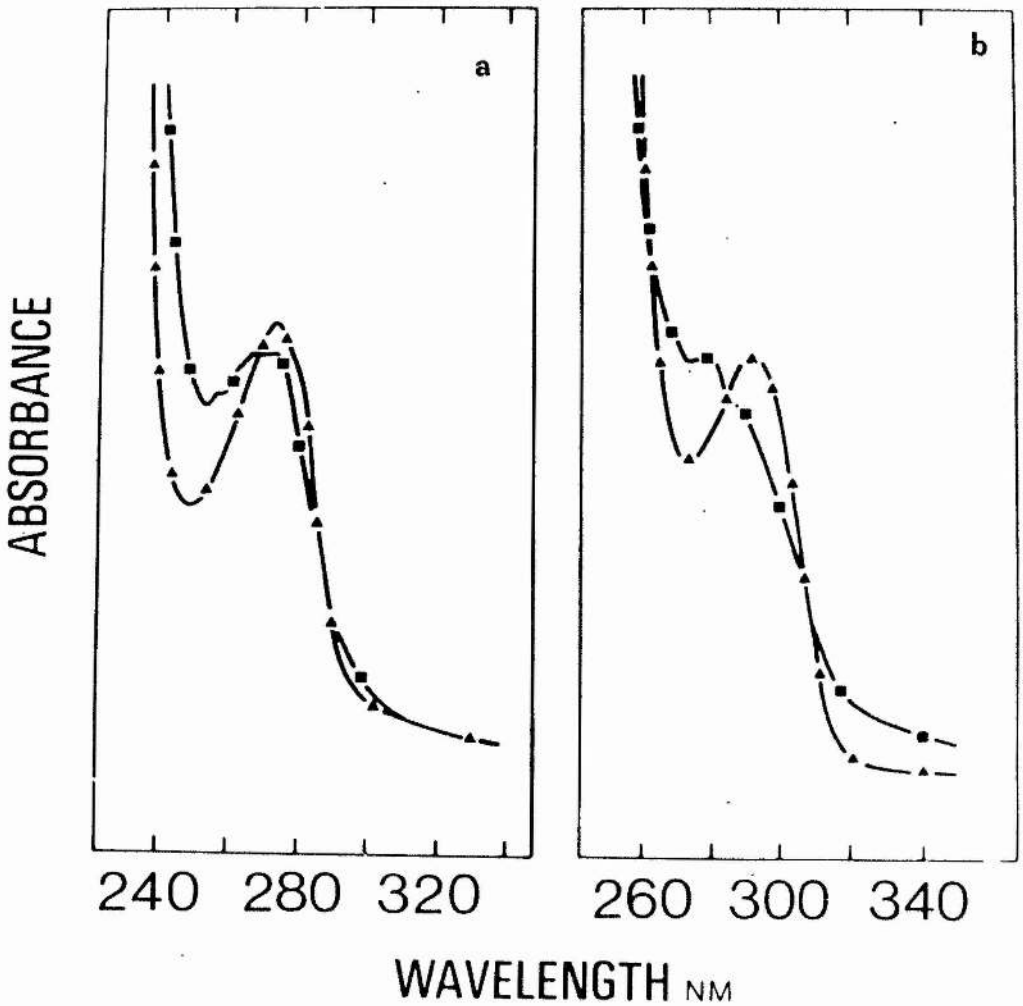
TABLE 13

AMINO ACID COMPOSITION OF SINGLE-TUBE FRACTIONS FROM THE ELASTASE-SODIUM DODECYL SULFATE DIGEST OF ELASTIC FIBRILS FROM TROUT BULBUS ARTERIOSUS

AMINO ACID	TUBE		
	13	26	35
Hydroxyproline	14.1	6.1	7.0
Aspartic acid	81.5	30.9	15.0
Threonine	69.3	26.0	25.9
Serine	98.1	31.3	17.7
Glutamic acid	91.7	36.2	25.9
Proline	92.6	74.5	96.3
Glycine	106.4	435.0	471.5
Alanine	140.3	184.0	149.3
Valine	57.9	47.3	49.2
Half-cystine	0.0	0.0	0.0
Methionine	20.3	6.8	4.2
Isoleucine	35.2	8.9	7.2
Leucine	62.9	32.9	33.3
Tyrosine	18.6	37.9	50.3
Phenylalanine	26.3	7.5	15.9
Hydroxylysine	0.0	0.0	0.0
Lysine	37.5	5.8	3.4
Histidine	14.8	3.7	5.9
Arginine	32.5	14.7	19.8
Lysinonorleucine*	0.0	1.2	1.0
Isodesmosine*	0.0	4.8	0.8
Desmosine*	0.0	4.5	0.4

Values are expressed as residues/1000 total amino acid residues

* Expressed as lysine equivalents



F I G U R E   32

The ultraviolet absorption spectra of the elastase digest of trout (0.5 mg of protein/1 ml) (▲) and bovine ligamentum nuchae (1 mg of protein/1 ml) (■) preparations in (a) 0.1 N HCl and (b) 0.1 N NaOH.

in alkaline conditions an emission maximum at 415 nm which was reduced in intensity but not shifted, at acid pH (156). In contrast, trout digests showed, under acid conditions, an emission maximum at 303 nm which was shifted to 345 nm and reduced in intensity at alkaline pH (Figure 33).

Mechanical properties of salmon purified bulbus arteriosus

A set of typical force-extension curves obtained with a specimen of salmon purified bulbus arteriosus is shown in Figure 34, which represents the behaviour of the material upon extension to 1.9 times its original length and subsequent slow relaxation. When the specimens were extended to rupture the breaking strain corresponded to 1.20. The stress-strain diagrams were almost linear and the Young's modulus was found to average $5.5 \pm 0.4 \cdot 10^5\ Nm^{-2}$.

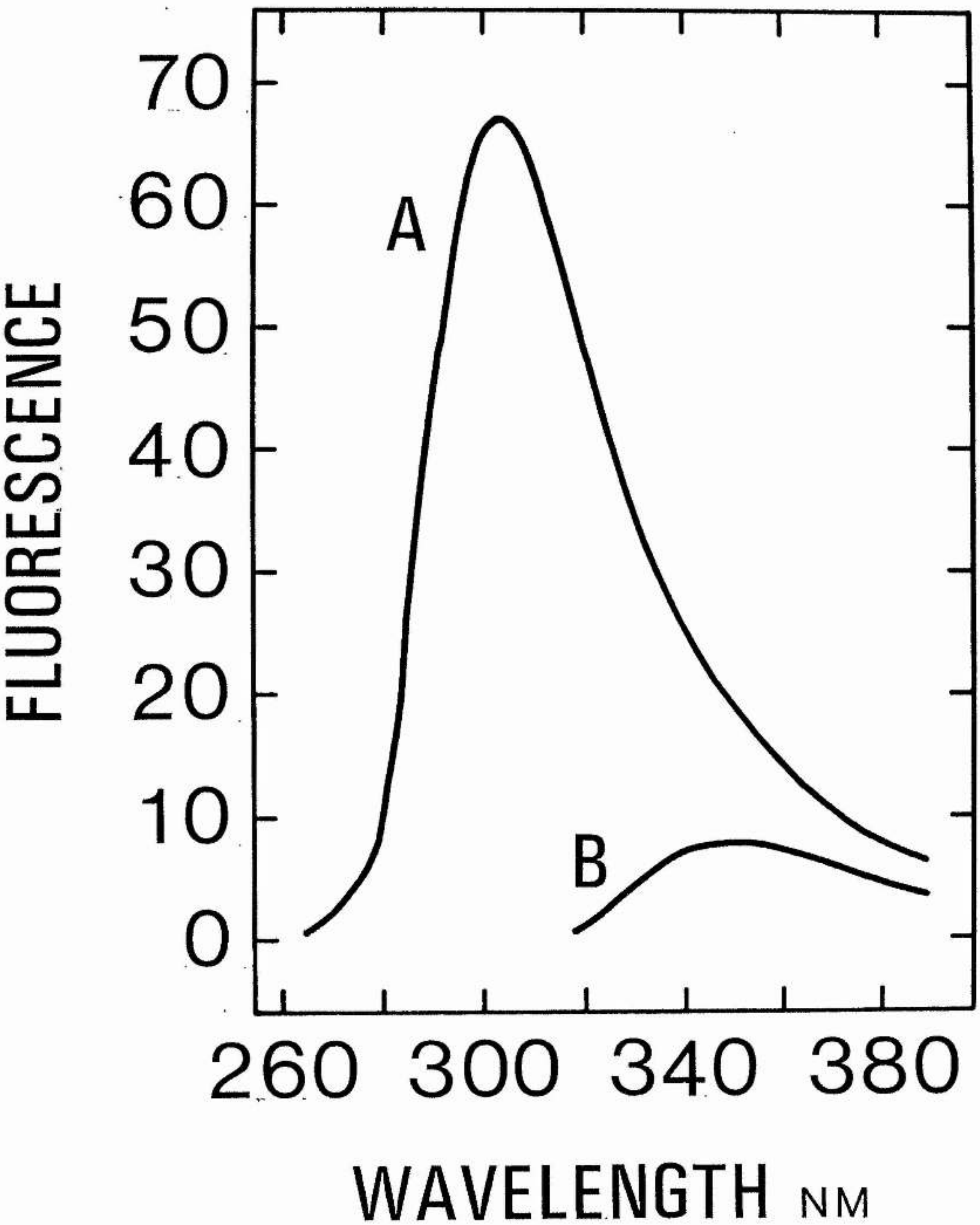
Wide-angle X-ray analysis

The wide-angle x-ray scattering patterns of salmon and trout bulbus arteriosus purified elastic fibrils were identical and showed broad amorphous rings corresponding to spacings of 9.8, 4.5 and 2.2 Å. Figure 35 shows the equatorial region and the corresponding microdensitometer trace of one such pattern obtained with salmon preparation.

FIGURE 33

The spectra of the fluorescent light emitted by the elastase digest of trout elastic fibrils in (A) 0.1 N HCl and (B) 0.1 N NaOH. Peptide concentration, 0.5 mg/ml. Activation at 253.7 nm.

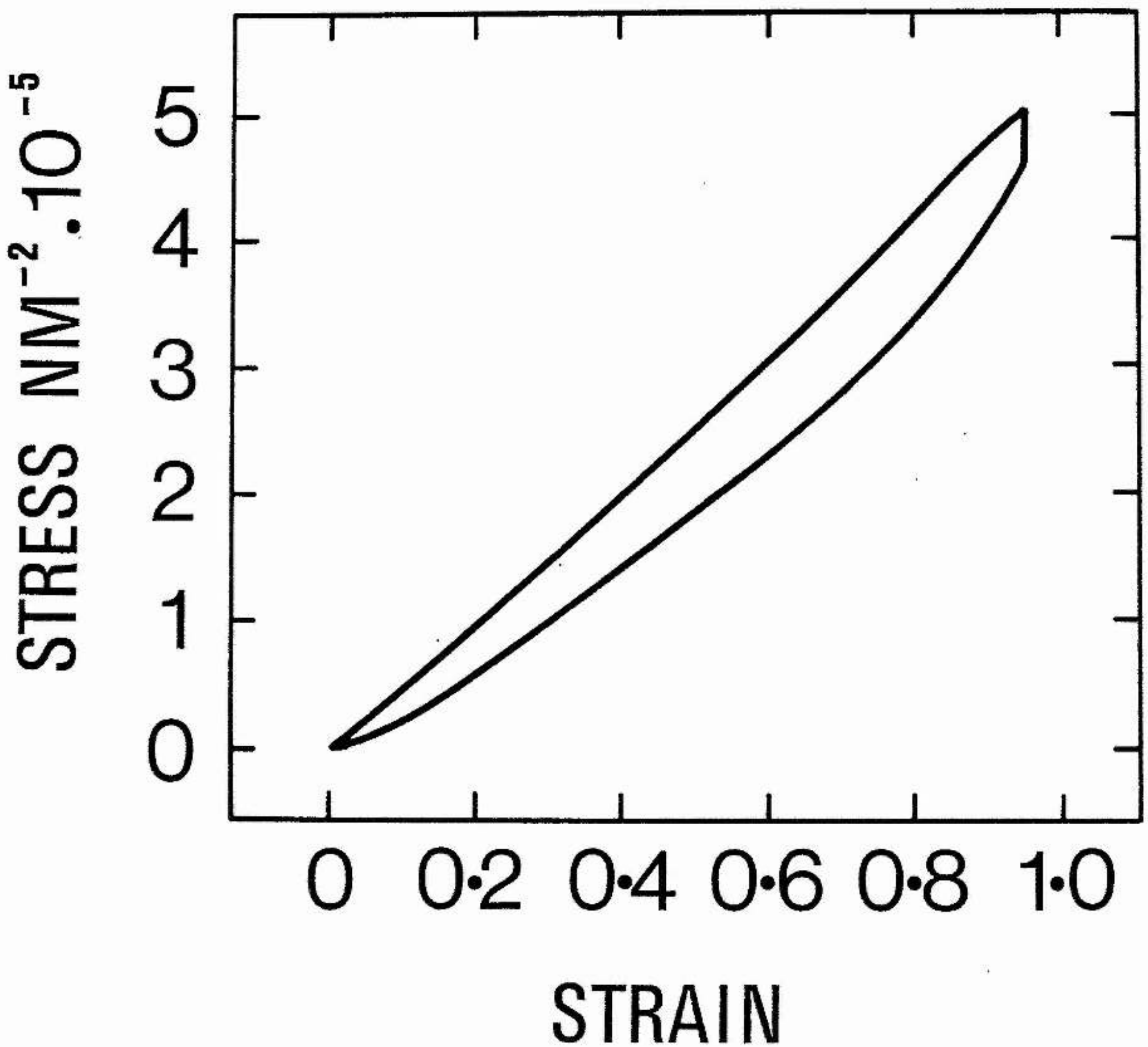
FIGURE 33


70
60
50
40
30
20
10
0
A
B
FLUORESCENCE
260 300 340 380
WAVELENGTH NM

F I G U R E 34

Stress-strain diagram of a purified specimen of salmon bulbus arteriosus during an extension-relaxation cycle. Arrowheads indicate the direction of path around the curve.

FIGURE 34

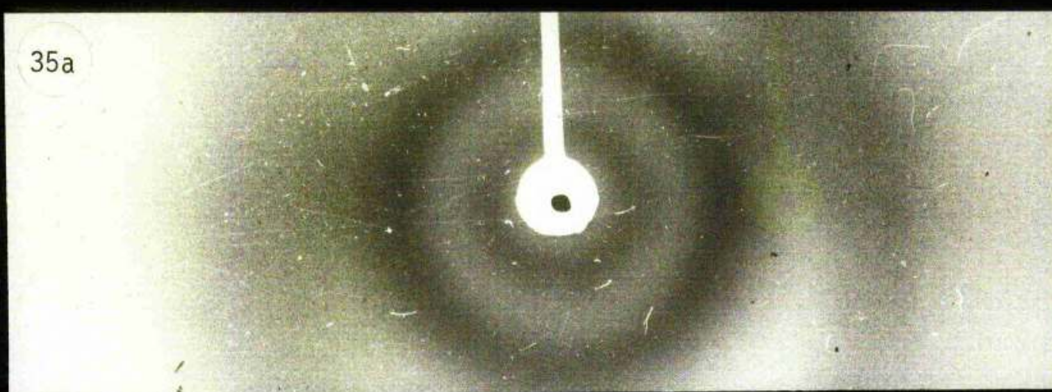


F I G U R E 35

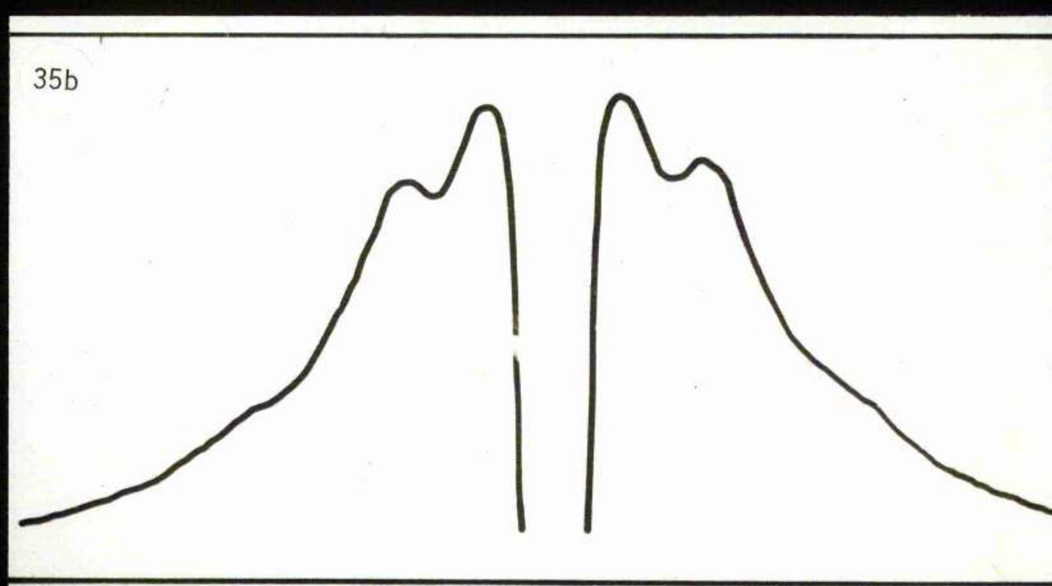
(a) The equatorial region of the wide-angle X-ray diffraction pattern of dry purified salmon bulbus arteriosus.

(b) Microdensitometer trace of the above diffraction pattern.

35a



35b



In negatively contrasted preparations, salmonid and mammalian elastin fibrils exhibited almost identical ultrastructural features. The fibers were, in fact, resolved into a lateral array of "beaded" primary filaments of about 2.5 nm in diameter, with shortest equatorial periodicities at 5.5 nm and 4.5 nm, respectively, which presumably represented interfilament distances, being within the range of values which can be directly estimated for this parameter on electron micrographs. It should be noted that an equatorial periodicity of between 4.6 and 5.0 nm has also been detected by low-angle X-ray analysis in a specimen of purified elastin from bovine ligamentum nuchae (14). Despite the beaded appearance exhibited by the primary filaments, optical diffractions did not reveal any significant meridional periodicity, a result which is supported by the absence of a meridional X-ray diffraction pattern in samples of ligamentum nuchae elastin (14).

When embedded material was examined, it was apparent that the two types of elastin differed markedly in their affinity for electron-dense cations and in their average fibril diameter. The dissimilarity in staining characteristics probably reflected the higher concentration of ionizable acidic groups present in the salmonid protein (about 13 residues /1000 total amino acid residues as compared with 4 in bovine elastin), as later discussed.

As far as fibril diameter is concerned, it is interesting to note that although the number of primary filaments which constitute a fibril appears to be fairly constant in salmonid elastin, as indicated by the uniformity of fibril diameter, nevertheless the protein has an inherent ability to undergo further aggregation. This aggregation was observed in the tissue where fibrils were aligned and in close contact over considerable distances and in purified preparations where an ordered arrangement was induced during centrifugation. In both these instances the fibrils fuse to form structures morphologically very similar to the fibrils of mammalian elastin. In fact, concomitant with the formation of fibrillar aggregates is a marked reduction in the affinity of the material for electron-dense cations. Although there is no experimental explanation for this phenomenon, it might be suggested that during aggregation, acidic groups, normally located at the surface of individual fibrils, may change their ion-exchange and ionization characteristics when transferred from an aqueous environment to a mainly hydrophobic domain. In this observation may lie the rationale behind the amorphous appearance, in conventionally stained thin sections, of mammalian elastic fibers, structures in which fibrils are normally aligned and closely packed.

Preparations of trout and salmon elastic fibrils exhibited amino acid compositions widely at variance with that of purified mammalian ligament elastin. There was a general enrichment in polar amino acid residues, which probably accounts for the differences in the staining properties of salmonid and mammalian fibrils observed by electron micrpscopy. It is, however, interesting to note that, in spite of a threefold increase in the concentration of dicarboxylic amino acids, 90% of these, as in the bovine protein, are in amide form. The total concentration of the four major nonpolar residues, viz., proline, glycine, alanine, and valine, is not greatly altered, but the relative proportions of these amino acids are changed, glycine being substantially enriched at the expense of alanine and particularly valine. In this respect it is worth noting that similar molar ratios between these residues have been reported by Sage and Gray (25) and Sandberg (157) in the elastins of other teleosts, purified by different techniques. Compositional differences between salmonid and mammalian preparations also involve both the overall concentration of crosslinking amino acids, which is considerably reduced, and the relative proportions of these residues. In the latter respect, it is interesting to note that in salmonids the major crosslink is the aldol condensation product rather than desmosine. Salmonid fibrils are also enriched in α-amino adipic acid δ-semialdehyde, recovered as ε-hydroxynorleucine. It should be noted, however, that the concentration of this lysine derivative when determined in the hydrolysate of the mammalian protein by a direct colorimetric assay was found to be considerably lower than that estimated by Lent et al. (21) using a radioactive labeling technique.

It is, of course, open to speculation wether these gross compositional differences merely reflect the inefficiency of the isolation procedure in removing contaminating proteins, particularly when it is considered that the susceptibility of salmonid elastic fibrils to collagenase digestion precluded the use of this enzyme as a final purification stage in their isolation. It should be noted, however, that the collagen content of the trout bulbus arteriosus is no more than 5% on a dry weight basis (Figure 28). The efficiency of Guan-HCl and DTE in solubilizing this minor component was indicated by (a) the absence of collagen fibrils in the purified preparations when these were extensively examined by electron microscopy, (b) the low hydroxyproline content of this material, and (c) by the fact that the composition of the collagenase digest residue is very close to that of the untreated preparation apart from a significant increase in the concentration of desmosines. The possibility of contamination by other tissue proteins has been tentatively assessed by the examination of peptides obtained after digestion of the trout preparations with elastase in the presence of sodium dodecyl sulfate. The only clear indication of the presence of one such contaminant was provided by the isolation of a high molecular weight fraction corresponding to 0.5% of the starting material and with an amino acid composition totally at variance with the rest of the preparation. Of greater significance is the fact that the peptide fraction eluted in tube 26 after agarose fractionation shows a considerably elevated crosslink content yet still exhibits an amino acid composition very close to that of the parent preparation. If,in fact, it is assumed that the contaminating material is chemically bound to the elastin component, presumably via aldehyde functions, it would be reasonable to expect that fractions which contain large proportion of crosslinking sites will also show above-average levels of contamination. It is unfortunate that the usefulness of such a technique in establishing the purity of the preparation is somewhat limited by the fact that only 50% of the starting material was subjected to fractionation on agarose, large losses having occurred prior to this chromatographic separation. This might be due to extensive degradation of the protein during enzymic digestion which coupled with the low crosslink content of the preparation lead to the production of large amount of low molecular weight material.

In spite of uncertainities concerning the composition of salmonid elastins, a significant divergence in primary structure between these proteins and that present in bovine ligament is clearly supported by the following evidence. In the first instance, the concentration of valine is so low that its normalization to levels typical of mammalian elastins would imply a contamination in excess of 60%, even by a protein devoid of this amino acid. Secondly, the prevalence of the aldol condensation product over desmosine and isodesmosine strongly suggests differences in those sequences carrying lysyl residues involved in the formation of crosslinks. Finally, the salmonid preparations differ from the bovine protein in their susceptibility to digestion, not only by elastase but also by a collagenase preparation from which activity toward bovine elastin had been removed by affinity chromatography.

The identification in trout and salmon elastin of primary filaments comparable in diameter to those present in the mammalian protein suggests that this mode of packing of polypeptide chains is a feature which has been acquired at a very early stage in elastin evolution. The conservation of this feature in proteins so dissimilar in primary sequence supports the view (14) that the primary filament is essential for the generation of the mechanical properties of the protein.

It should be noted that in salmon elastin fibrils, as in bovine ligament preparations,the response to stress is linear and reversible with only limited hysteresis. This latter feature, in itself, suggests the absence of a large contamination by an inert protein. The Young's modulus was lower than that exhibited by bovine elastin but compatible with the crosslink concentration. It is worth noting that the value of 16300 for the mean intercrosslink chain molecular weight (Mc) calculated from dynamometry is, notwithstanding the uncertainty inherent in such determination (158), very close to that of 17000 calculated from the crosslink content. The formula adopted for the evaluation of Mc from tensile strength measurements (153) requires that the macromolecules comprise random flexible chains crosslinked in a three-dimensional network. That salmonid elastin satisfies the first condition is indicated by its wide-angle X-ray diffraction pattern which is similar to that of mammalian ligament elastin (14, 87) in exhibiting only three broad rings which are common to many proteins in random conformation (159). In addition, as pointed out elsewhere, the identification of primary filaments in negatively stained specimens is not incompatible with the existence of a three-dimensional arrangement of polypeptide chains in a reticulum of finite dimensions (160). Equally, the evaluation of Mc from analytical data presupposes that the only crosslinks operative in the structure are the lysine-derived polyfunctional amino acids reported in Table 12. In view of the high tyrosine content of salmonid preparations it would not be unreasonable to suspect the presence of additional crosslinks, such as di- and tri-tyrosine, which stabilize the network structure of resilin, another elastomeric protein found in insects. However, neither absorption nor fluorescence spectra indicated the presence of such crosslinks in the proteins under investigation.

Two additional observations concerning the relationship between elastin and other connective tissue components may be worth of consideration. In mammalian tissues elastin fibrils are usually associated with a fine reticulum of microfibrillar component which has been implicated in the biogenesis of elastin (9, 161). Such a reticulum was,however, absent in the tissue under investigation. Equally lacking was the specific spatial relationship found in mammalian elastic ligaments (85). In salmonid elastic tissue collagen appears, by virtue of its disposition, to be unable to work as a strain limiter. Its function might be to anchor the vessel to adjacent tissues.

As a concluding remark, it is interesting to note that considerable compositional variations affecting not only most of the amino acids but also the lysine-derived crosslinks are not accompanied by any substantial alteration of those parameters which are directly linked to the mechanical properties of the material. This lends weight to the proposal that the basic requirement for elastic recoil is the kinetic freedom of the polypeptide chains (162) as in such a system even a substantial compositional shift will not impair the mechanical functioning of the protein, provided that it does not induce any considerable folding of the polypeptide chains into stable secondary conformations.

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